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Alteration of Gene Expression during the Induction of Freezing Tolerance in *Brassica napus* Suspension Cultures[1]

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ANNE M. JOHNSON-FLANAGAN[2] AND JAS SINGH*
Plant Research Centre, Agriculture Canada, Ottawa, Ontario K1A 0C6, Canada

ABSTRACT

Brassica napus **suspension-cultured cells can be hardened to a lethal temperature for 50% of the sample of −20°C in eight days at room temperature with abscisic acid. During the induction of freezing tolerance, changes were observed in the electrophoretic pattern of [^{35}S]methionine labeled polypeptides. In hardening cells, a 20 kilodalton polypeptide was induced on day 2 and its level increased during hardening. The induction of freezing tolerance with nonmaximal hardening regimens also resulted in increases in the 20 kilodalton polypeptide. The 20 kilodalton polypeptide was associated with a membrane fraction enriched in endoplasmic reticulum and was resolved as a single spot by two-dimensional electrophoresis.** ***In vitro*** **translation of mRNA indicate alteration of gene expression during abscisic acid induction of freezing tolerance. The new mRNA encodes a 20 kilodalton polypeptide associated with increased freezing tolerance induced by either abscisic acid or high sucrose. A 20 kilodalton polypeptide was also translated by mRNA isolated from cold-hardened** ***B. napus*** **plants.**

During the attainment of freezing and hyperosmotic tolerance, plant cells undergo metabolic changes including increases in proteins, amino acids, membrane lipids, RNA, ABA, and soluble sugars (28). The plasmalemma undergoes compositional (31) and qualitative modifications (18) during hardening. Despite extensive observations of changes in plasmalemma behavior during freezing and hyperosmotic stress in hardened plant cells (30), a direct link between the metabolic changes and the amelioration of extracellular freezing injury has not been established. Furthermore, the trigger initiating these changes in plant cells genetically capable of hardening is not known.

One approach to determining the genetic basis of freezing tolerance is the identification of the products from genetic messages; protein changes may reflect such a modification. Previous studies have demonstrated increased concentration of soluble proteins during hardening (1, 26). Increases in soluble proteins having apparent mol wt of 30 and 25.5 kD in *Brassica napus* cv Goreczanski (12) and 240 and 115 kD in wheat (25) have been reported, while little change has been reported in alfalfa (5) and rye (1). While some of these studies show a correlation between polypeptide changes and the induction of freezing tolerance, they do not provide insight into the role of these changes in the mechanism of freezing tolerance. The plasma membrane is considered as a primary site of freezing injury (30). On this basis, Uemera and Yoshida (31) examined the plasma membrane polypeptides in hardened and nonhardened rye plants and found few differences. It has been suggested that the ER may play a role in the alteration of the plasma membrane during hardening in plant cells. Therefore, polypeptide differences in the ER during the induction of freezing tolerance could be of significance.

Sarhan and Chevrier (26) began examining genetic control of low temperature acclimation of winter wheat through a study of the regulation of protein synthesis at the transcriptional level. They quantitated the activities of RNases and RNA polymerases, DNA levels, RNA levels, and synthesis of DNA and RNA. However, Guy *et al.* (7) were the first to provide direct evidence of altered gene expression during low temperature exposure. They observed the synthesis of unique mRNAs during the early stages of low temperature acclimation of spinach. One of these messages encoded a polypeptide present in *in vitro* labeled plants. However, it is not clear whether the new mRNAs result from low temperature exposure during acclimation or are required for the expression of freezing tolerance.

The present study utilized a microspore-derived embryogenic culture of *Brassica napus* cv Jet Neuf. This culture can be hardened to a LT_{50}[3] of −20°C in 8 d at room temperature with ABA (20). Therefore, it is a useful system to study *in vivo* protein labeling during the induction of freezing tolerance. This study examines the developmental sequence of protein synthesis, accumulation and turnover during the attainment of freezing tolerance, and determines the subcellular location of the polypeptides of interest. The genetic basis for freezing tolerance is also studied. Unique mRNA is identified by *in vitro* translations in cell free systems. These are compared to *in vitro* translation products from cold-hardened and nonhardened plants.

MATERIALS AND METHODS

Plant Material. Winter rape (*Brassica napus* cv Jet Neuf) microspore-derived suspension cultures were prepared and cultured as previously described (20). Induction of freezing tolerance was carried out by culturing cells for 8 d at 25°C in medium containing 13% (w/v) sucrose, 50 μg ABA (20). Nonhardened cells were cultured for 8 d in medium containing 6.5% (w/v) sucrose, minus ABA. Cold-hardened plants were vernalized at 4°C under a 16h/8h day/night photoperiod for 12 weeks. Nonhardened plants were grown at 20°C under the same photoperiod until leaf size and number were similar to the vernalized plants.

Freeze-Test. Freeze-testing of the cells was performed as described by Orr *et al.* (20). Two ml aliquots of cell suspensions were collected, washed three times with 10 ml water, pelleted, and resuspended in 3 ml water and divided into 0.5 ml aliquots in 16 × 100 mm tubes. Cells were incubated at 0°C for 30 min

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[2] Present address: Department of Plant Science, University of Alberta, Edmonton, Alberta, T6G 2P5, Canada.

[3] Abbreviations; LT_{50}, lethal temperature for 50% of the sample, PMSF, phenylmethylsulfoxyl fluoride.

in a Lauda K4/R cooling bath and freezing was initiated at −2.5°C by inoculation with dry ice. The temperature was lowered step-wise by 2.5°C every 30 min. Samples were allowed to thaw at 4°C for 20 h. Survival was assayed by the uptake of fluorescein diacetate (33).

Protein Labeling. [^{35}S]Methionine (specific activity > 1000 Ci/mmol) (Dupont) was added to the cell suspensions to give a final concentration of 50 μCi/ml. On d 2 following subculturing and the addition of ABA, cells were incubated with [^{35}S]methionine for 2 h, washed three times with the corresponding supernatant from nonlabeled cell suspensions, and resuspended in the same supernatant. An aliquot from each treatment was collected and freeze-dried after the initial 2 h labeling and on d 4, 6, and 8. Sampling was repeated exactly as outlined above, on d 4, 6, and 8, using cold supernatants from similar cultures to wash the cells. All steps were carried out aseptically.

Heat Shock. Cells were incubated with shaking at 38°C for 2 h. [^{35}S]Methionine was added to a final concentration of 10 μCi/ml at the start of the incubation period. Cells were washed in three volumes of 13% (w/v) or 6.5% (w/v) sucrose buffered with 50 mM K-phosphate at pH 6.5 and freeze-dried.

SDS Slab PAGE. Lyophilized cells were ground in 500 μl 100°C SDS-solubilizing buffer for 30 s then transferred to a boiling water bath for 1 min. *In vitro* translation products were boiled in the SDS-solubilizing buffer for 1 min. The solubilizing buffer consisted of 12% glycerol (w/v), 2% SDS (w/v), 5% mercaptoethanol (v/v), and 0.001% bromophenol blue (v/v) in 62.5 mM Tris-HCl adjusted to pH 6.8 (31). Soluble proteins were separated from membrane bound proteins by grinding lyophilized cells for 30 s in 400 μl ice-cold medium consisting of 6.25% mercaptoethanol (v/v), and 1 mM PMSF in 50 potassium phosphate buffer, adjusted to pH 6.5, and centrifuged at 105,000*g* for 30 min. SDS was added to the supernatant to a final concentration of 2% (v/v), followed by the addition of glycerol to 12% (v/v), giving a final volume of 500 μl.

The proteins were analysed on 1.5 mm-thick, 12%, 15% or 7 to 15% linear gradient polyacrylamide gels with a Tris-glycine buffer system (13). Samples of 25 μg protein or labeled protein equivalent to 2×10^5 cpm was loaded and electrophoresis was performed at 16°C with a constant current of 35 mamp per slab. The gels were stained in freshly prepared 50% (w/v) TCA and 0.1% Coomassie blue-R-250 (19) and destained in 30% methanol and 7% glacial acetic acid. Gels for detection of glycopeptides were electro-blotted (Transphor, Hoefer products) on to nitrocellulose membranes at 100 V for 45 min in 0.192 M glycine, 0.025 M Tris (pH 8.3), and 20% (v/v) methanol. The nitrocellulose blots were then autoradiographed using Kodak X-Omat AR film (14).

Isolation and Characterization of Membrane. Cells from 8 d hardened or nonhardened cultures were ground with a mortar and pestle for 2 min in a grinding medium consisting of 50 mM Tris, 0.25 M sucrose, 3 mM EDTA, 1 mM EGTA, and 5 mM mercaptoethanol, adjusted to pH 7.5 with HCl. The ratio of cells to grinding medium was 2:1 (v/v). The volume was made up to 10 ml with grinding medium and the slurry was centrifuged for 10 min at 14,500*g* in a Sorvall SS-34 rotor. The pellet was washed with 10 ml grinding medium and spun at 14,500*g*. The pellet was retained and the supernatants were pooled. Following centrifugation of the supernatant for 20 min at 144,000*g* in a Beckman Ti-60 rotor, the pellet was resuspended in 2 ml grinding medium, and layered on either a continuous 15 to 45% (w/v) sucrose gradient, or a step-wise gradient containing 1 ml 15%, 3 ml 25%, 3 ml 36% and 3 ml 43% sucrose. Both gradients were prepared with grinding medium and layered on a 1 ml 50% sucrose cushion. The tubes were centrifuged at 139,000*g* for 2 h at 4°C in a Beckman SW-40 rotor.

The pellet from the 14,500*g* centrifugation (above) was resuspended in a small amount of grinding buffer and layered on a 0.6 M and 1.6 M sucrose step gradient and centrifuged for 1 h at 30,000*g* in a Beckman SW-40 rotor (11). The band at the interphase was enriched in mitochondria. All operations were carried out at 0 to 4°C. The magnesium-shift experiments for identifying ER were modified from Ray (23). All steps were carried out as described above, except that the grinding medium was modified by the deletion of EDTA and EGTA and the inclusion of $MgCl_2$ to 6 mM.

Antimycin-A-insensitive NADPH Cyt *c* reductase activity was determined spectrophotometrically by measuring Cyt *c* reduction at 550 nm (9). Cyt *c* oxidase activity was determined by measuring oxidation of Cyt *c* at 550 nm (9). Protein was determined by the method of Sedmak and Grossberg (27).

One- and Two-Dimensional Electrophoresis of Membrane Proteins. Cells were labeled with [^{35}S]methionine on d 2 and membranes were prepared on a step gradient as described above. The band was collected, rapidly diluted to 35 ml in water, pelleted at 144,000*g* for 12 min in a Beckman Ti-60 rotor, and solubilized in the solubilizing buffer for one-dimensional analysis. Gels were stained with Coomassie blue, electroblotted on to nitrocellulose for glycopeptide staining as described above or fluorographed. For two-dimensional analysis, membranes were solubilized in a lysis buffer containing 9.5 M urea, 2% (v/v) Nonidet P-40, 2% ampholytes (0.8% pH 5–7, 0.8% pH 4–8, and 0.4% pH 3–10 [Biorad]) and 5% (v/v) mercaptoethanol, or in the solubilizing buffer followed by an excess of the lysis buffer. Two-dimensional gels were prepared and run according to the method of O'Farrell (19). Gels were stained with Coomassie blue as described above, or silver stained according to the methods of Merril *et al.* (17) or fluorographed.

RNA Isolation. Extraction of total cellular RNA was performed according to Colbert *et al.* (2): fresh or freeze dried cells or leaves were ground with sand in a mortar and pestle in 4 M guanidine thiocyanate, 50 mM Tris-HCl (pH 7.6), 2% (w/v) lauroyl sarcosine (sodium salt), and 1% mercaptoethanol. The ratio of reagent to sample was 2:1 (v/v). The slurry was centrifuged at 3,000*g* for 10 min and the pellet was discarded. The supernatant was made to 2.4 M CsCl, layered on a 2 ml cushion of 5.7 M CsCl in 0.1 M EDTA, and centrifuged in a Beckman SW-40 Ti-rotor at 209,000*g* for 18 h at 7°C. DNA was collected from the top of the cushion. The RNA pellet was then treated according to Maniatis *et al.* (16). Following purification, the RNA was desalted by passing it through a spun-column of Sephadex G-50 (Med) equilibrated in water (16). Poly (A^+) mRNA was isolated with Hybond-MAP (Amersham).

DNA and RNA Determination. DNA and RNA was determined by absorbance at 260 nm with an OD of 1 taken as 50 μg/ml DNA and 40 μg/ml RNA. The 260/280 was 1.7 to 1.8 for DNA and 1.8 to 2.0 for RNA.

Polysome Isolation. Polysomes were prepared according to the method of Verma and Marcus (32) with minor modifications. Cells frozen in liquid N_2 were ground in a mortar and pestle in 6 ml of extraction buffer containing 200 mM Tris-HCl (pH 8.5), 400 mM HCl, 250 mM sucrose, 5 mM mercaptoethanol, 0.5% Nonidet P-40, 50 mM Mg-acetate, and 25 mM EGTA. The homogenate was centrifuged at 20,000*g* for 10 min in an SS-34 rotor. The postmitochondrial supernatant was layered on 2.5 ml of 1.5 M sucrose in 200 mM Tris-HCl (pH 8.5), 200 mM KCl, and 50 mM Mg-acetate and centrifuged for 90 min at 105,000*g* in a Beckman SW-40 Ti rotor. The pellet was suspended in water. All steps were carried out at 4°C.

***In Vitro* Translation.** Translations were performed with rabbit reticulocyte lysate or a wheat germ extract translation kit (DuPont). Approximately 100 to 200 ng Poly A^+ or polysomes containing 1 to 2 μg RNA was added to the standard reaction volume. The rabbit reticulocyte lysate assay contained 10 μl

lysates, 5 μl [^{35}S]methionine (1000 Ci/mmol), 5.5 μl amino acid cocktail, 2 μl 1 M K-acetate, and 32.5 mM Mg-acetate in a total volume of 25 μl. The reaction was allowed to proceed for 1 h at 37°C and the incorporation was monitored on the basis of TCA-precipitable counts. At the termination of the reaction, aliquots were mixed 1:1 (v/v) with the electrophoresis solubilizing buffer, and electrophoresed as described above. The wheat germ extract assay contained 5 μl, wheat germ extract, 5 μl [^{35}S]methionine (1000 Ci/mmol), 4 μl amino acid cocktail, 0.5 μl creatine kinase (0.125 mg/ml), 1 μl RNase A inhibitor, 1 μl 3.75 M K-acetate, and 1 μl 50 mM Mg-acetate in a final volume of 25 μl. The reaction was allowed to proceed for 2 h at 22°C and incorporation was measured as described above.

Scintillation Counting. Aliquots of the SDS-solubilized protein were counted in 5 ml Aquasol (DuPont).

Protein Determination. Protein was determined by the method of Peterson (22). Protein standards for mol wt determination were BSA, ovalbumin, aldolase, carboxypeptidase A, chymotrypsinogen, ribonuclease A, and Cyt *c*.

RESULTS

Changes in Polypeptide Profiles during Hardening. The degree of freezing tolerance attained by the cultured *B. napus* suspension cells at d 2, 4, 6, and 8 of hardening is presented in Figure 1. After 8 d, cells began to decline in hardness (20). Coomassie blue staining of total cell extracts failed to detect differences in polypeptide profiles or their relative proportions during the induction of freezing tolerance. However, fluorographs from cells collected every 2 d showed distinct differences in the polypeptide patterns (Fig. 2). In gels of hardening cells, there was an increasing intensity of the 17 and 20 kD polypeptides over the 8 d period. The 17 kD polypeptide also appeared in smaller quantities in the nonhardened cells. Minor differences in 32.5 and 92 kD bands may also have occurred over the 8 d period.

Protein profiles from cells, labeled for 2 h on d 4, 6, and 8 were similar to cells labeled on d 2. There was no major redistribution of the label. The most significant difference was the induction of a 20 kD polypeptide on d 8. Occasionally, fluorographs showed faint bands of approximately 96, 18, and 16 kD in both hardened and nonhardened 2-d-old cells. These bands were never apparent by d 6. Freezing tolerance could also be attained, albeit to a lesser degree, using either a combination of 6.5% sucrose and ABA or 13% sucrose alone in the culture media (20). Cells hardened by these alternative regimes also showed labeling of the 17 and 20 kD polypeptides when examined by SDS-PAGE.

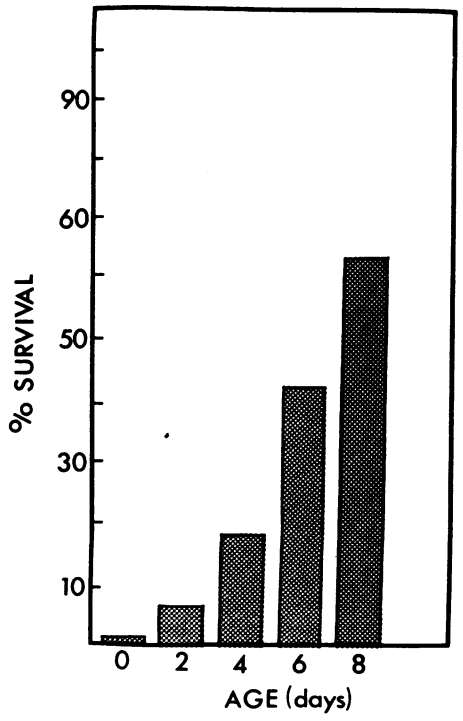

FIG. 1. Development of freezing tolerance in *B. napus* suspension-cultured cells. Freezing tolerance was determined as survival after freezing to −20°C, as described in "Materials and Methods."

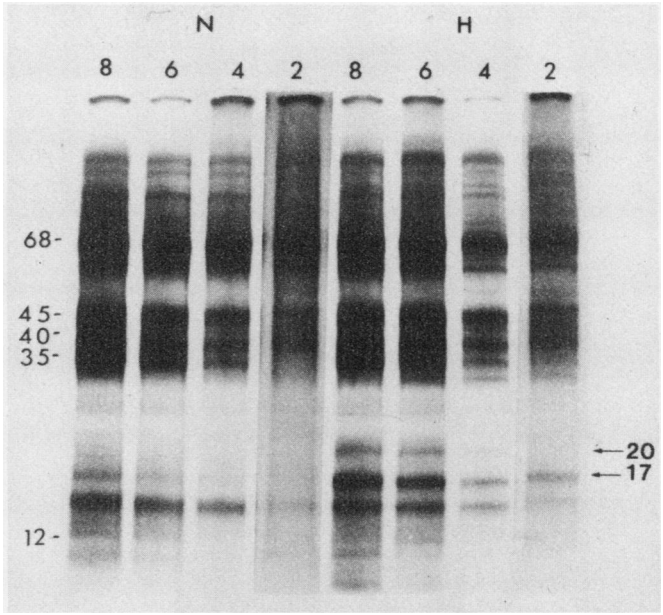

FIG. 2. Fluorograph of one-dimensional gel of polypeptides from *B. napus* suspension-cultured cells during the induction of freezing tolerance. Mol wt are shown on the left side of the fluorograph. N, nonhardened; H, hardened. All cells were labeled 2 d after subculturing. Changing bands are indicated on the right side of the fluorograph. Numbers indicate days of culture. Fluorograph was overexposed to show 17 and 20 kD polypeptides clearly.

Electrophoresis of the Soluble Polypeptide Fraction. SDS-PAGE of the polypeptides initially extracted in phospate buffer showed the presence of the 17 kD band in the soluble fraction. Although most of the low mol wt polypeptides were soluble, the 20 kD polypeptide was membrane bound (not shown).

Heat Shock Polypeptides. The fluorographs from electrophoretic profiles of 8-d hardened and nonhardened cells were compared to the heat shock polypeptides to determine whether the proteins induced during hardening were stress proteins (7). Only two major heat shock polypeptides corresponding to 16 and 74 kD were apparent in cells from 8-d cultures. However, gel profiles of cell cultures from 2-d (Fig. 3) to 6-d cells contained bands corresponding to 96, 83, 74, 56, 51, 40, 34, 30, 28, 20, 28, and 16 kD. In the absence of heat shock, polypeptide labeling profiles were similar to 6-d and 8-d lanes in Figure 2. Heat shock did not cause an immediate increase in freezing tolerance, nor did it alter the induction of freezing tolerance over the normal 8-d hardening period.

Preparation and Identification of an ER-Enriched Fraction. Microsomal membranes isolated from 8-d cells separated into three bands and a pellet on sucrose density gradient centrifugation (Fig. 4). Assays for antimycin-insensitive NADPH Cyt *c* reductase activity and Cyt *c* oxidase activity (Table I) indicated the highest enrichment of ER in band 2 of both hardened and nonhardened preparations. Addition of magnesium causes a

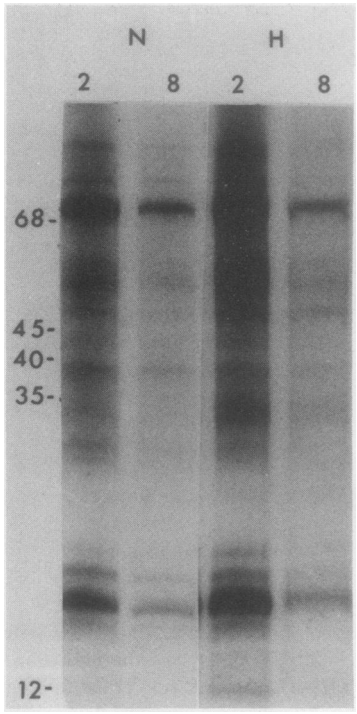

FIG. 3. Fluorograph of heat-shocked hardened and nonhardened *B. napus* suspension-cultured cells. Mol wt are shown on left side of fluorograph. N, nonhardened; H, hardened. Heat-shock treatment is described in "Materials and Methods." Numbers indicate days of culture.

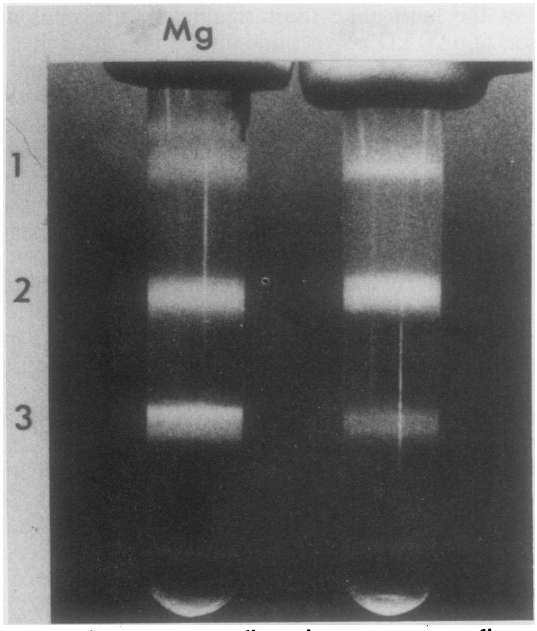

FIG. 4. Banding pattern on a discontinuous sucrose gradient of membranes from hardened *B. napus* suspension-cultured cells. Mg, medium containing magnesium. Bands 1, 15/25%; 2, 25/36%; 3, 36/43%.

major portion of this band to shift to a higher buoyant density suggesting the presence of ER (Fig. 4). Antimycin-insensitive NADPH Cyt *c* reductase activity also demonstrated a shift from band two to band three (Fig. 5).

Subcellular Location of the 20 kD Polypeptide. Both Coomassie-stained gels and fluorographs from hardened cells show the presence of the 20 kD polypeptide in bands 2 and 3 of the sucrose density gradient (Fig. 6). The high level of Cyt *c* oxidase activity

Table I. *Enzyme Activities of Membrane Bands, from Hardened and Nonhardened B. napus Suspension-Cultured Cells, Separated by Sucrose Density Gradient Centrifugation*

Values are the mean of three separate preparations each containing 5 ml of packed cells.

Cells	Enzyme Activity	
	Antimycin A-insensitive NADPH Cyt *c* reductase	Cyt *c* Oxidase
	$\mu mol \cdot min^{-1} \cdot ml^{-1}$ *of packed cells*	
Hardened		
1 (15/25%)	0.06 ± 0.003	0.01 ± 0.002
2 (25/36%)	0.10 ± 0.019	0.12 ± 0.020
3 (36/43%)	0.02 ± 0.001	0.09 ± 0.030
Nonhardened		
1 (15/25%)	0.04 ± 0.003	0.01 ± 0.004
2 (25/36%)	0.06 ± 0.002	0.06 ± 0.010
3 (36/43%)	0.02 ± 0.005	0.05 ± 0.015

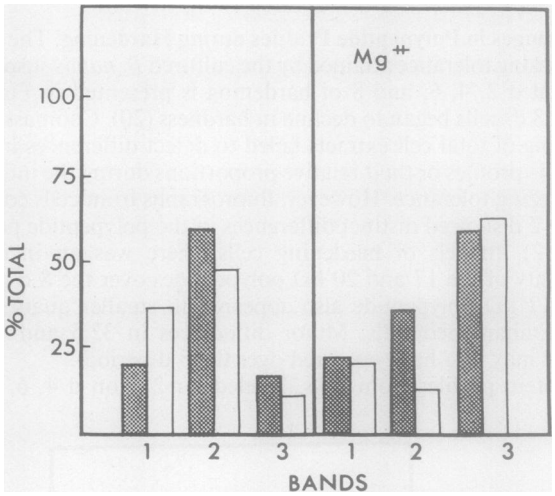

FIG. 5. Magnesium-shift of protein content and antimycin-insensitive NADPH Cyt *c* reductase activity in membrane bands for hardened suspension-cultured cells. Bands 1, 15/25%; 2, 25/36%, and 3, 36/43%. Shaded bars represent protein content and empty bars represent NADPH Cyt *c* reductase activity.

in these bands suggested mitochondrial contamination. However, the 20 kD polypeptide was not present in a gradient-enriched mitochondrial fraction. Small amounts of a 20 kD polypeptide were observed in the nonhardened cell preparation.

Two-Dimensional Electrophoresis of the ER-Enriched Fraction. The ER-enriched fraction was solubilized in either the SDS solubilizing buffer followed by the urea/Nonidet P-40 solubilizing buffer or the urea/Nonidet P-40 solubilizing buffer, directly. The results of the latter are shown in Figure 7. In both cases, the 20 kD polypeptide was resolved as a single spot.

RNA/NDA Ratios. The total RNA/DNA ratio was very low on d 2 in both cell cultures (Fig. 8). The ratio increased markedly in the hardened cells by d 5, but decreased thereafter. In contrast, the ratio increased gradually, in nonhardened cells reaching approximately the same proportion as the hardened cells by d 8. Total RNA levels were significantly higher in 8 d hardened cells in comparison to the 8 d nonhardened cells (210 ± 29 μg/flask *versus* 108 ± 33 μg/flask). This increase, however, was not reflected in the efficiency of *in vitro* poly A^+ directed incorporation.

***In Vitro* Translation of Poly(A^+) mRNA.** Poly(A^+) mRNA from hardened and nonhardened cells directed *in vitro* transla-

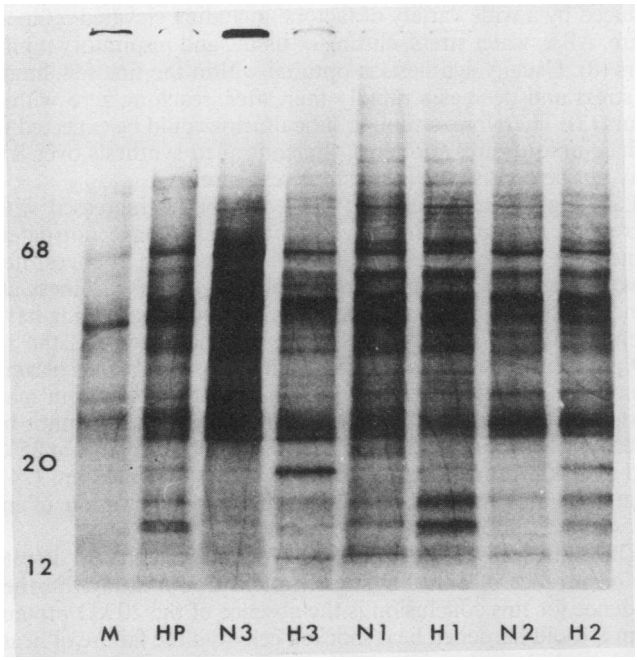

FIG. 6. Fluorograph of one-dimensional gel of polypeptides from ER-enriched membranes and mitochondria of hardened and nonhardened *B. napus* suspension-cultured cells. Membranes were separated on a sucrose step gradient and assayed and mitochondria were prepared as described in "Materials and Methods." Lanes are labeled as follows: N, nonhardened; H, hardened; HP, hardened pellet; M, mitochondria. Numbers refer to bands in Figure 5.

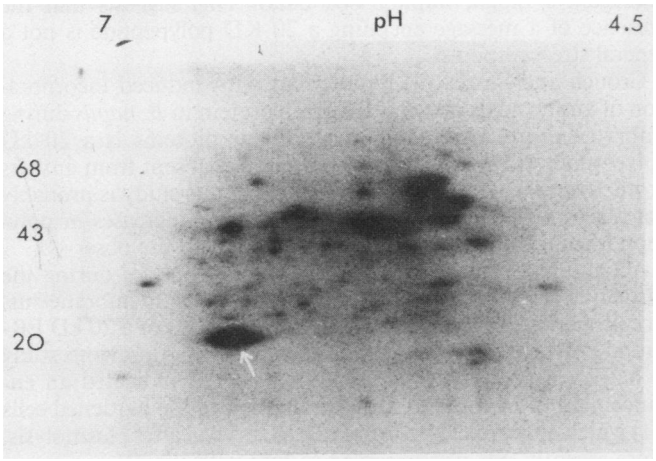

FIG. 7. Fluorograph of a two-dimensional gel of polypeptides from ER-enriched membrane fraction of hardened *B. napus* suspension-cultured cells. Membranes were separated on a sucrose step gradient, band 2 + 3 was taken as the ER-enriched fraction, and was solubilized and electrophoresed in two dimensions, as described in "Materials and Methods."

tions of polypeptides over a wide range of mol wt in either the rabbit reticulocyte or wheat germ systems (Fig. 9). However, polypeptide with an apparent mol wt of 20 kD was present only in *in vitro* translation of mRNA isolated from hardened cells. The new mRNA was present 2 d of subculturing into the hardening medium, but did not appear to increase in abundance during the 8 d hardening period. Message encoding a 17 kD polypeptide was also present in hardened cells and to a lesser extent in nonhardened cells. One-dimensional SDS electrophoresis profiles in the *in vitro* translation products from polysomes

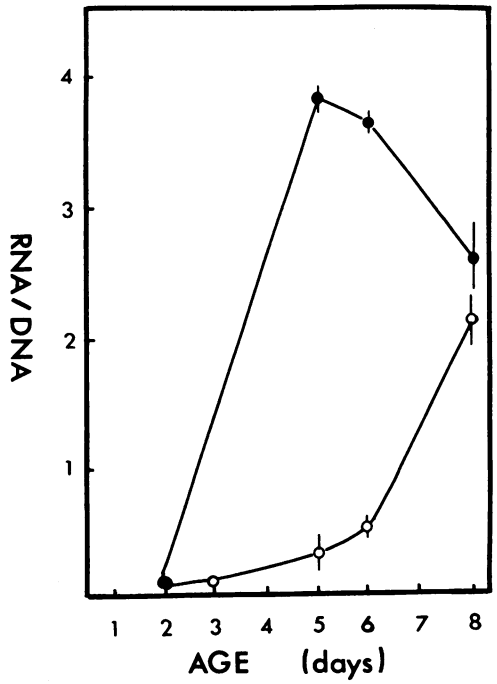

FIG. 8. RNA/DNA ratios during the induction of freezing tolerance in *B. napus* suspension-cultured cells. DNA and RNA were isolated and quantitated according to "Materials and Methods." (●), Hardened cells; (○), nonhardened cells. Values are the mean of at least three separate preparations ± SE.

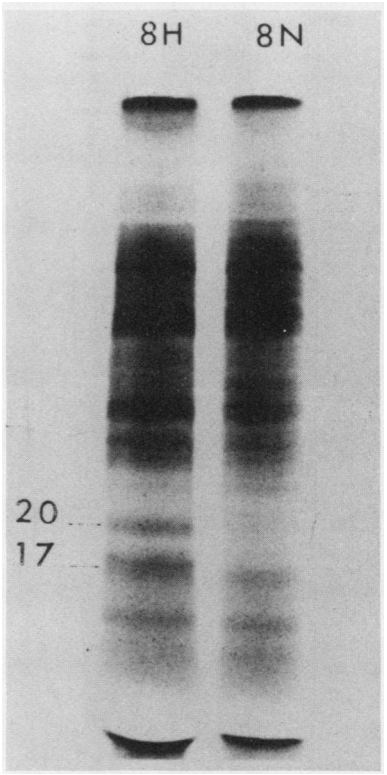

FIG. 9. Fluorograph of *in vitro* translation products from Poly A^+ of hardened and nonhardened cells. Equivalent counts were added to each lane. H, hardened; N, nonhardened. Mol wt is shown on the left side.

were the same as those from translation of poly(A^+) mRNA.

***In Vitro* Translation of Plant mRNA.** Poly(A^+) mRNA from *B. napus* plants grown at 4°C for 8 to 12 weeks encoded a wide range of polypeptides in the rabbit reticulocyte system (Fig. 10). These include polypeptides with apparent mol wt of 20 and 17 kD. The 20 kD polypeptide was not present in mRNA from plants grown at 25°C (nonhardened). Hardened plants showed an LT_{50} of below −15°C.

DISCUSSION

There were no significant differences between Coomassie stained profiles of total proteins extracted from hardened and nonhardened cultured cells in this study. This is in agreement with observations in rye (1) and alfalfa (5). However, protein changes have been identified with Coomassie staining of gels of acetone extracts of hardened Brassica plants (12).

Fluorographs of labeled polypeptides showed numerous changes in their SDS-gel profiles during hardening in cultured *Brassica* cells. The significance of these changes can be determined by comparisons with polypeptide profiles from nonhardened cells, cells hardened in 6.5% sucrose and ABA, cells hardened in 13% sucrose, and heat-shocked cells. The rationale for these controls is as follows. Changes in nonhardened cells indicated normal synthesis related to culture age while changes under the two alternative hardening regimes indicate synthesis in response to ABA and increased osmoticum, respectively. The heat shock polypeptides indicate a general stress response (8) that is not related to the induction of freezing tolerance.

Following subculturing, cells from all regimes produced unique stress polypeptides corresponding to 18 and 16 kD. The synthesis of these decreased as the cultures aged. Stress proteins can be induced by a wide variety of factors, including elevated temperature, ABA, water stress, cutting of tissue, and respiratory inhibitors (8). Usually synthesis is optimal within the first few hours of stress and decreases rapidly thereafter, reaching zero within hours (3). Therefore, although subculturing could be expected to induce unique stress proteins, the continued synthesis over 8 d may not be consistent with a stress response.

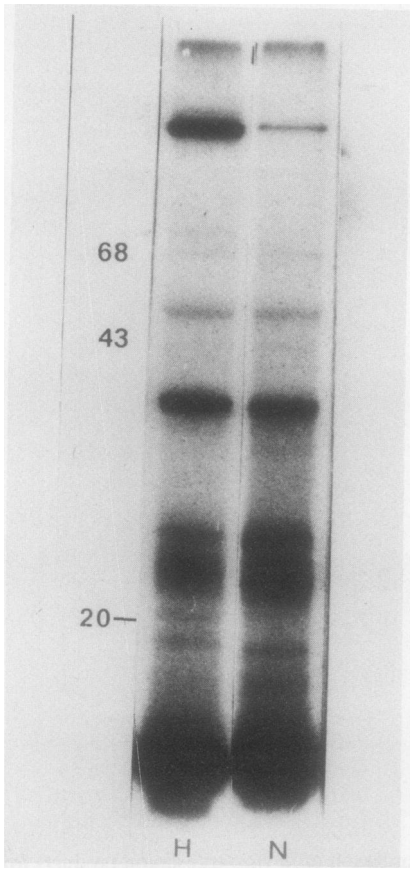

FIG. 10. Fluorograph *in vitro* translation products from Poly A^+ of cold-hardened and nonhardened plants. Mol wt shown on the left side.

Labeling of the 17 and 20 kD polypeptides increased with increasing freezing tolerance. Previous studies have not determined the temporal development of polypeptide changes, nor have changes been compared with the degree of hardiness attained. Increases of a 17 kD polypeptide during hardening have been reported previously (24, 31). In cold hardened rye, the 17 kD polypeptide was observed in stained gels of isolated plasma membrane (31). However, the possibility that this protein may have been trapped in the plasma membrane vesicles cannot be ruled out. Brome grass cultures were hardened with ABA at 25°C and a 17 kD protein accumulated in the culture medium (24). Therefore, this may be consistent with our observation of increases in a soluble 17 kD polypeptide.

The 20 kD polypeptide is probably not a stress protein despite the occurrence of a 20 kD stress protein in our study. Further evidence for this conclusion is the absence of the 20 kD protein from 8-d-old hardened heat shocked cells and the failure of heat-shock to induce freezing tolerance. Furthermore, the cultures could be hardened at 2°C for 4 weeks in the absence of ABA (minus ABA, minus benzyladenine, 6.5% sucrose). The degree of hardening, however, was not as high as in the presence of ABA at 25°C (20% survival *versus* 50% survival at −20°C, respectively). *In vitro* translation of poly(A^+) isolated from cold-hardened cells showed the distinct presence of a 20 KD polypeptide on one-dimensional SDS-PAGE (W Orr, AM Johnson-Flanagan, J Singh, unpublished data). This suggests that the presence of a message encoding a 20 KD polypeptide is not a general stress response.

Crouch and Sussex (4) reported an ABA-induced incorporation of amino acids into a 12S storage protein in *B. napus* during embryo culture. One component of this proteins is a 20 kD polypeptide. However, this 12S protein was absent from anthers or microspores. The 20 kD polypeptide in this study is probably not a subunit of the 12S protein because no increases in polypeptides equivalent to the other four subunits were observed.

Accumulation of membrane bound polypeptides during the hardening period may indicate alteration of the membrane surface properties. In the present study, the amount of a 20 kD ER-bound polypeptide increased during hardening. A previous study in hardened and nonhardened *B. napus* cells indicated an enhancement of membrane strand formation in the hardened cells (11) which appeared to be correlated to survival after plasmolysis. More recently, ER was observed to fuse with the plasma membrane during strand formation in hardened rye and *Brassica* cells (29). Intrinsic differences in ER properties may be responsible for this phenomenon.

Although the plasma membrane has been implicated as a site of freezing injury, plasma membrane renewal during the induction of freezing tolerance has been observed to be mediated by a proliferation of ER vesicles and their fusion with the plasmalemma (18). Observation of polypeptide changes associated with an ER enriched fraction reported here is consistent with the role of the ER during hardening.

Changes in RNA/DNA ratios and total RNA in *B. napus* grown in the presence of ABA and high sucrose indicate that there is increased RNA and DNA synthesis during the induction of freezing tolerance in the absence of low temperature. Cold acclimation in plants has been associated with increased RNA (15, 21, 26, 28), while DNA contents may increase modestly (26) or may not increase (6, 15). Increased DNA content has been

suggested to represent increased copies of rDNA (21, 26), because there is increased rRNA content during cold acclimation (6, 21). In the present study, rRNA was also observed to increase during hardening. These changes would facilitate the increased rate of protein synthesis during hardening reported by numerous workers (15, 26).

The mRNA population changed rapidly in response to the hardening medium. Within 2 d, mRNA encoding a unique polypeptide with an apparent mol wt of 20 kD was present in the hardening cells. Although labeling of the 20 kD and a 17 kD polypeptide increased over the hardening period, the amount of mRNA encoding these polypeptides did not increase in the present study. Guy *et al.* (7) also observed little change in the amount of unique mRNA during cold acclimation of spinach. At the present time, there is no unequivocol data to suggest that the 20 and 17 kD polypeptides produced by *in vitro* translation is identical to the polypeptides of similar mol wt obtained by labeling experiments.

The 20 kD polypeptide produced by *in vitro* translation is a unique gene product in hardened cells and its synthesis seemed to be controlled at the transcription level. Tseng and Li (personnal communications) observed the presence of a message, translating a 20 kD polypeptide in ABA hardened potato stem cultures. Guy *et al.* (7) observed a number of unique translation products in cold hardened spinach. However, only one of these, with an apparent mol wt of 82 kD, was present in *in vivo* labeled plants.

A message encoding a 20 kD polypeptide appeared concomitantly with the induction of freezing tolerance in *B. napus*. *In vitro* translation products from plants suggested that a message encoding a 20 kD polypeptide was also present in hardened plants. At the same time, a 20 kD polypeptide was also labeled *in vivo* in cells undergoing hardening. Experiments are in progress to elucidate the physiological role of the polypeptide(s) in the induction of freezing tolerance in *B. napus*.

While this work was being completed, Meza-Basso *et al.* (1986 Plant Physiol 82: 733–738) reported the observation of polypeptide changes in *Brassica napus* germinating seedlings subjected to growth at low temperature for 48 h. Results cannot be comparable to the data here because their low temperature exposure is more representative of a short-term low temperature treatment rather than a long-term hardening regime.

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