Altered gene expression during cold acclimation of spinach

(mRNA/in vitro translation/stress proteins/freezing tolerance)

C. L. Guy†, Kevin J. Niemi‡, and Robert Bramble§

Departments of †Plant Pathology and ‡Horticultural Science, The University of Minnesota, St. Paul, MN 55108

Communicated by Paul J. Kramer, January 31, 1985

ABSTRACT Exposure of spinach (Spinacia oleracea L.) plants to a constant 5°C induced a greater tolerance to extracellular freezing. The metabolic basis of this cold acclimation response in plants is not understood. In this study we tested the hypothesis that cold acclimation derives from altered gene transcription. We found that exposure of plants to low temperature resulted in a rapid and stable change in the translatable poly(A)+ RNA populations extracted from leaves, as determined by a cell-free in vitro translation assay. The initial appearance of mRNAs for two high molecular weight translation products correlated with an increase in freezing tolerance. Cold acclimation of plants for 8 days resulted in further qualitative changes in mRNA populations. At least four additional mRNAs increased in concentration upon continued exposure of spinach to 5°C, whereas three other mRNAs present in 20°C-grown leaves decreased. We also tested the possibility that the low temperature-induced mRNAs might encode heat shock proteins. We studied heat shock-induced protein synthesis by in vivo labeling techniques and found that spinach synthesized at least eight distinctive heat shock proteins during exposure to 40°C. Most polypeptides induced by exposure to low temperature, however, appeared not to be heat shock proteins. Thus, the change in mRNAs induced by low temperature is a separate response from that induced by high temperature.

The increase in freezing tolerance of plants during cold acclimation has been proposed to result from a combination of physiological changes and metabolic alterations that depend on altered gene expression (1). A number of earlier studies described the changes in physiology, metabolism, or enzyme activity associated with the induction of greater freezing tolerance (2). Several of these studies demonstrated an appearance and disappearance of certain membrane proteins (3, 4) and changes in the isozyme composition of a number of enzymes (5–10). Although evidence does exist for altered gene expression in other types of plant stress responses (11, 12), direct evidence of altered gene expression during cold acclimation has not been presented.

We report here data that indicate a qualitative alteration in the population of translatable mRNA during cold acclimation in spinach. We have extracted RNA from nonacclimated and cold-acclimated spinach leaves, prepared and translated in vitro the mRNA fraction, and by electrophoresis the translation products found that development of increased freezing tolerance is correlated with the rapid appearance of two new, continuously expressed species of mRNA.

MATERIALS AND METHODS

Plant Material and Cold Acclimation. Leaves of spinach, Spinacia oleracea L. cv. Bloomsdale (Northrup King), were used for all studies. Plants were grown in a controlled environment growth chamber with an air temperature at plant height of 20 ± 1°C in the light and 17 ± 1°C in the dark. Illumination (12 hr/day) was provided by cool white fluorescent tubes and supplemented with incandescent lighting as described (10). Plants were watered daily and half-strength Hoagland's nutrient solution (13) was provided each week.

At 5 weeks after planting, half of the plants were transferred to a second growth chamber for cold acclimation. The air temperature at plant height was maintained at a constant 5 ± 1°C during the cold acclimation period. Light and dark periods were 12 hr each.

Cold Hardiness Assessment. Freezing tolerance was determined by freezing detached leaves in a controlled temperature bath (10). After equilibration of the leaf tissue at 0°C, the bath temperature was lowered at a rate of 2°C/hr. Extracellular freezing of the leaf tissue was initiated by the addition of a small chip of ice to the tissue at −1°C. Leaf samples were removed at 1°C intervals from −3°C to −16°C and the tissue was thawed at 4°C overnight. Injury was determined by visual observation of permanent water soaking and by measurement of electrolyte loss by changes in electrical conductivity (10). Leaves darkenened by water in the intercellular spaces 24 hr after thawing were considered injured; disks from this frozen tissue that showed 50% leakage of electrolytes were considered killed.

Total RNA Isolation. Total RNA from nonacclimated and cold-acclimated spinach leaves was prepared by a modified phenol/chloroform method (14, 15). Leaf tissue was detached, rinsed with distilled water, and blotted dry. The tissue (20 g) was minced with a razor blade and homogenized in 50 ml of extraction buffer (100 mM Tris-Cl, pH 8.8/100 mM NaCl/5 mM EDTA/1% N-lauryl sarcosine/40 units of heparin per ml) with a Polytron homogenizer for five 1-min periods with 1-min cooling intervals on ice. Buffer-saturated phenol/chloroform/isoamyl alcohol, 50:48:2 (50 ml), prepared with freshly distilled phenol and containing 0.5% 8-hydroxyquinoline, was added to the tissue extract and shaken for 10 min at room temperature. The emulsion was centrifuged at 6000 × gsv for 10 min at 10°C. The aqueous phase was removed and extracted three additional times with an equal volume of buffer-saturated phenol/chloroform/isoamyl alcohol. The RNA was precipitated overnight at −20°C after the addition of 2.5 vol of cold (−20°C) ethanol. The RNA precipitate was collected by centrifugation and washed with cold 70% ethanol. Residual ethanol was evaporated and the RNA was resuspended in 10 ml of 3 M sodium acetate, pH 5.5/5 mM EDTA (16). RNA was pelleted by centrifugation (6000 × gsv for 10 min at 0°C) and this sodium acetate wash step was repeated twice. The RNA pellet was dissolved in sterile, nuclease-free water and stored at −80°C.

Isolation of Poly(A)+ RNA. Total RNA isolated from spinach leaves was diluted with distilled water to a concentration of 1 μg/μl, heated to 70°C for 5 min, and rapidly cooled

$\text{Present address: Ornamental Horticulture Department, University of Florida, Gainesville, FL 32611.}$

$\text{To whom reprint requests should be addressed.}$
on ice. The sample was adjusted to 0.5 M NaCl by the addition of 1/3 vol of 2 M NaCl in chromatography buffer (10 mM Tris-HCl, pH 7.6/5 mM EDTA/0.5% N-lauroyl sarcosine) and applied to an oligo(dT)-cellulose (Collaborative Research) affinity column (1-mL bed volume) at a rate of 6 ml/hr (17). The column bed was washed with 12 ml of chromatography buffer containing 0.5 M NaCl, and poly(A)⁺ RNA was then eluted with 10 ml of chromatography buffer without NaCl. The RNA was precipitated overnight at -20°C after addition of 0.1 vol of 2.5 M sodium acetate (pH 5.5) and 2.5 vol of cold ethanol. This RNA fraction was subjected to a second cycle of oligo(dT)-cellulose chromatography to further enrich the poly(A)⁺ RNA for subsequent use in the in vitro translation experiments.

**In Vitro Translation.** Rabbit reticulocyte lysates (Bethesda Research Laboratories or Promega Biotec, Madison, WI) were programmed with 1 µg of spinach poly(A)⁺ RNA in the presence of 50 µCi (1 Ci = 37 GBq) of [³⁵S]methionine with a specific activity of 1460 Ci/mmol (Amersham) (18). The reaction volume was 30 µl and RNasin (Promega Biotec) was added to a concentration of 1 unit/µl. The reaction (90 min at 30°C) was terminated by the addition of an equal volume of double-strength NaDodSO₄ buffer (120 mM Tris-HCl, pH 6.8/2%/NaDodSO₄/2%/dithiothreitol/20%/glycerol).

**In Vivo Labeling of Proteins.** Leaf tissue from nonacclimated and cold-acclimated plants was rinsed with sterile distilled water, and thirty 5-mm (diameter) disks were excised and transferred to 1 ml of sterile distilled water in a sterile test tube. The disks were vacuum infiltrated with distilled water and incubated in a controlled environment chamber with illumination at 20°C (nonacclimated) or 5°C (cold-acclimated) for 2 hr before the addition of the radiolabeled amino acid. Proteins were labeled for 4 hr with 50 µCi of sterile [³⁵S]methionine. Nonradioactive methionine was added to a concentration of 1 mM for a chase of 5 min to terminate the labeling interval. The leaf disks were rinsed with distilled water and homogenized with a 2-ml glass tissue grinder in 1 ml of NaDodSO₄ buffer (60 mM Tris-HCl, pH 6.8/1%/NaDodSO₄/1%/dithiothreitol/10%/glycerol). The extract was boiled for 5 min and centrifuged at 15,000 × g, for 10 min at 4°C, and the supernatant fluid was removed and stored at -60°C.

**Heat Shock Protein Synthesis.** Nonacclimated plants grown at 20°C day/17°C night were placed in a controlled environment incubator and incubated at 25°C, 30°C, and 35°C sequentially for 2 hr at each temperature. Leaf disks were then removed from the plants as described above, and the disks were transferred to 40°C for 1-hr heat shock and then labeled with [³⁵S]methionine for 1 additional hr at 40°C. Proteins were extracted as described above and separated by polyacrylamide gel electrophoresis.

**Electrophoresis.** Protein samples containing equal quantities of acid-insoluble radioactivity from [³⁵S]methionine, radiolabeled in vivo or in vitro, were applied to one-dimensional 10%, 12%, or 15% polyacrylamide gels containing 1% NaDodSO₄ and subjected to electrophoresis with a constant current of 25 mA. The gels and buffer solutions were prepared as described (19). The maximal amount of [³⁵S]methionine-labeled protein that would not cause electrophoretic distortion was loaded onto the gels. After fixation and staining with Coomassie blue, gels were dried and exposed to Kodak XAR-5 film at -80°C. Proteins standards (purchased from Sigma) and their approximate molecular weights (∗10⁶) were myosin (205), phosphorylase B (97.4), bovine serum albumin (66), ovalbumin (45), carbonic anhydrase (29), soybean trypsin inhibitor (20.1), and cytochrome c (12.1).

**RESULTS**

**Induction of Freezing Tolerance.** When spinach plants are transferred from a growth temperature of 20°C to a tempera-

---

**Table 1. Effect of growth temperature on the freezing tolerance of spinach leaves**

<table>
<thead>
<tr>
<th>Days of treatment</th>
<th>Nonacclimated</th>
<th>Cold-acclimated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>-6</td>
<td>-6</td>
</tr>
<tr>
<td>1</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>-5</td>
<td>-9</td>
</tr>
<tr>
<td>3</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>-6</td>
<td>-10</td>
</tr>
<tr>
<td>5</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>-6</td>
<td>-10</td>
</tr>
<tr>
<td>7</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>-6</td>
<td>-10</td>
</tr>
<tr>
<td>14</td>
<td>-10</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>-7</td>
<td>-11</td>
</tr>
<tr>
<td>21</td>
<td>-12</td>
<td></td>
</tr>
</tbody>
</table>

*Five-week-old plants were exposed either to nonacclimating temperatures (20°C day/17°C night) or to cold-acclimating temperatures (5°C day/5°C night) for various lengths of time before leaves were removed and their freezing tolerance was determined.*

---

**FIG. 1.** **In vitro** translation products of mRNA isolated from leaf tissue of plants exposed to nonacclimating temperatures (20°C day/17°C night) or cold-acclimating temperatures (5°C/5°C) for various lengths of time. Translation products were labeled with [³⁵S]methionine and electrophoretically separated in a NaDodSO₄/15% polyacrylamide gel. Lanes: A, products of endogenous mRNA of the rabbit reticulocyte lysate; B, control leaf tissue from 5-week-old plants; C, E, G, and I, leaf tissue exposed to nonacclimating temperatures for 2, 4, 8, and 16 days; D, F, H, and J, leaf tissue exposed to cold-acclimating temperatures for 2, 4, 8, and 16 days. Molecular weight markers are shown as ∗10⁶. Arrowheads denote major low temperature-induced polypeptides.
into protein by 10-fold over the background incorporation of the reticulocyte lysates. The translation products were analyzed by one-dimensional NaDodSO₄/polyacrylamide gel electrophoresis and autoradiography. Exposure of spinach leaves to 5°C resulted in the appearance of several new translatable mRNA species not present in plants grown at 20°C. New mRNAs for two prominent high molecular weight polypeptides with Mr's of 82,000 and ~180,000 (Fig. 1) are present after only 2 days and persist up to 16 days of cold acclimation. The mRNAs for these two high molecular weight proteins appear not to show any further increase in concentration between 2 and 16 days. Several other changes were also observed in the mRNA populations of leaf tissue later during cold acclimation. In addition to the appearance of the two high molecular weight in vitro translation products, at least four other mRNAs encoding polypeptides with Mr's of 72,000, 43,000, 31,000, and 19,000 appear, whereas three other mRNAs for polypeptides with Mr's 68,000, 23,000, and 13,000 disappear following 8 days of cold acclimation (Fig. 2).

**In Vivo Protein Synthesis.** During the early stages of cold acclimation incorporation of [35S]methionine into leaf protein was very low at 5°C (Table 2), but as cold acclimation progressed the leaf tissue became more efficient in the incorporation of radiolabeled methionine into proteins at low temperature. After 16 days of exposure to 5°C the rate of incorporation by cold-acclimated leaf tissue at 5°C was equal to that of nonacclimated tissue labeled at 20°C. When leaf disks from cold-acclimated plants were labeled at 20°C instead of 5°C, more than twice as much label was incorporated into protein than by nonacclimated leaf tissue labeled at 20°C, and improved autoradiograms were obtained. Fig. 3 shows the pattern of protein synthesis in leaf disks from nonacclimated and cold-acclimated plants labeled immediately after transfer to 20°C. The same qualitative

<table>
<thead>
<tr>
<th>Treatment</th>
<th>20°C dpm × 10⁻⁴ per dish per hr</th>
<th>5°C dpm × 10⁻⁴ per dish per hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonacclimated</td>
<td>7.27 100</td>
<td>2.39 33</td>
</tr>
<tr>
<td>Cold-acclimated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 days</td>
<td>2.09 29</td>
<td></td>
</tr>
<tr>
<td>4 days</td>
<td>2.53 35</td>
<td></td>
</tr>
<tr>
<td>8 days</td>
<td>3.08 42</td>
<td></td>
</tr>
<tr>
<td>16 days</td>
<td>16.31 224</td>
<td>7.44 102</td>
</tr>
</tbody>
</table>

Percent incorporation is based on that of nonacclimated tissue labeled at 20°C.

patterns of proteins were synthesized in cold-acclimated leaf tissue at 20°C as at 5°C. Since the number of in vivo translation products greatly exceeded the resolving capacity of the one-dimensional gel system, only major changes resulting from cold acclimation were detected. Nevertheless, the pattern of protein synthesis in nonacclimated and cold-acclimated leaf tissue is clearly different. Several proteins present in cold-acclimated leaf extracts of Mr's 110,000, 82,000, 66,000, 55,000, and 13,000 were not found in nonacclimated leaf extracts. The Mr 82,000 band corresponds in electrophoretic mobility to the band observed in the in vitro translation products of mRNA from cold-acclimated leaf tissue, but the other bands with Mr's 110,000, 66,000, 55,000, and 13,000 are not apparent in the autoradiograms of the in vitro translation products. Excision of the leaf tissue and vacuum infiltration in distilled water resulted in a wound response that elicited the synthesis of two wound proteins (Fig. 3).

**Heat Shock Proteins.** Exposure of spinach to a heat shock of 40°C altered the pattern of protein synthesis. The synthesis of normal cellular proteins was sharply reduced, and eight heat shock proteins with Mr's of 107,000, 85,000, 74,000, 31,000, 27,000, 23,000, 21,000, and 19,000 were induced (Fig. 4).
3. Two of these heat shock proteins, with Mₜₛ of 31,000 and 19,000 have identical electrophoretic mobilities in in vitro translation products from mRNA induced by cold acclimation.

**DISCUSSION**

The increase in tolerance to extracellular freezing evoked by prior exposure to low, nonfreezing temperatures (cold acclimation) is common in plants. The development of freezing tolerance in plant tissues that results from cold acclimation is associated with many changes in both physiological functions as well as shifts in cellular biochemistry. The cold acclimation process is typically thought to require several weeks to promote freezing tolerance. Our results, however, show that exposure to cold-acclimating temperatures leads to a rapid increase in the hardness of spinach leaves that begins as early as 1 day and continues through the third day. An additional 18 days of exposure to 5°C were required to reach the maximal freezing tolerance of ~12°C. This increase in freezing tolerance during the initial stages of cold acclimation (which was first observed in spinach by Anne Fennell, personal communication) is much more rapid than many other workers have assumed.

Exposure of spinach to cold-acclimating temperatures results in an equally rapid change in the translatable mRNA population (Fig. 1). mRNAs for two high molecular weight translation products are present by 2 days of cold acclimation and persist at least through the 16th day of exposure to 5°C. Since our earliest RNA sampling time was 2 days after initiation of cold acclimation, the new species of mRNAs may appear even sooner after the transfer of the plants to cold-acclimating temperatures. The presence of the mRNAs for the high molecular weight polypeptides at 2 days of cold acclimation, when the freezing tolerance is rapidly increasing, and their persistence during the entire time the plants are exposed to 5°C suggest a possible relationship of de novo gene transcription to the increase in freezing tolerance.

Other changes in translatable mRNA populations resulting from low temperature exposure of spinach were observed by 8 days of cold acclimation. Several mRNAs for lower molecular weight translation products are newly synthesized or increase in concentration upon continued exposure to 5°C, while certain other mRNAs present in nonacclimated plant tissue disappear when spinach is maintained at 5°C (Fig. 2). Similar changes in translatable mRNA populations in plants have been shown to occur in studies of hormonally regulated physiological and developmental processes (15, 21-24). Such changes were hypothesized to occur during cold acclimation in plants (1) but have not been demonstrated previously. That changes in translatable mRNAs resulting from exposure of spinach to 5°C were resolved with a relatively low-resolution one-dimensional NaDodSO₄ gel system suggests that a number of other differences may exist in the mRNA contents of nonacclimated and cold-acclimated spinach leaves, and use of a two-dimensional electrophoretic system may resolve many more changes in mRNA translation products. Since in vitro translation results in the synthesis of polypeptides that presumably have not been post-translationally modified, changes in the in vitro protein synthesis patterns should reflect changes in gene expression. The appearance of new mRNA species during cold acclimation suggests a possible transcriptional control of gene expression during cold acclimation.

Changes in protein synthesis patterns resulting from different growth temperatures were more difficult to determine by in vitro labeling of spinach leaf disks. Low uptake of exogenous, labeled amino acid at low temperature and the need to wound the leaf tissue by excision to promote reasonable amino acid uptake complicated the analysis. Nevertheless, we did observe a protein band of M, 82,000 from cold acclimated leaf tissue (Fig. 3), which may correspond to one of the major in vitro translation products. Others also have found differences in protein electrophoretic patterns between nonacclimated and cold-acclimated tissues (3-10), but these earlier studies have not correlated these changes with the appearance of new species of mRNA. One consequence of exposure of spinach to low temperature may be the development of more active or more efficient protein synthesis at these low temperatures (Table 2).

Our work in this laboratory has determined that Neurospora crassa synthesizes proteins after a freezing stress that are similar to proteins this organism synthesizes during heat shock (unpublished data). Since plants synthesize a specific set of proteins when heat shocked (12), we examined the possibility that the cold-acclimation polypeptides are related to the heat shock proteins of spinach. We found that, upon exposure to heat shock conditions, spinach leaf tissue synthesized eight distinct polypeptides ranging from M, 107,000 to 19,000. The heat shock proteins were dissimilar in electrophoretic mobility to most of the polypeptides induced by cold acclimation (Fig. 3). This result indicates that the change in the population of mRNAs during cold acclimation is a separate response from that of heat shock (12) and represents an additional type of temperature response in plants.

The in vivo labeling technique used in this study caused a wound response that promoted the synthesis of two of the higher molecular weight heat shock proteins. Similarly, wounding of maize mesocotyl tissue promotes heat shock protein synthesis (24). These observations demonstrate the need to consider potentially confounding shifts in protein synthesis patterns resulting from incidental wounding of plant tissues during preparation for experimental purposes.

In summary, we propose that exposure of spinach to a temperature of 5°C alters the relative abundance of specific mRNAs and leads to an increase in the tolerance to extracellular freezing. The change in mRNAs during exposure to 5°C may result from expression of genes involved in adjustment of tissue metabolism to a low, nonfreezing temperature or these new mRNAs may encode proteins that are involved directly in the development of increased freezing tolerance. A greater understanding of the role that altered gene expression plays in cold acclimation and freezing stress tolerance will require isolation of the genes for the low temperature-induced mRNAs and identification of the encoded polypeptides and their functions.

We thank Anne Fennell for providing unpublished data on freezing tolerance of spinach and Nora Plesofsky-Vig for helpful discussion and critical reading of this manuscript. This research was supported in part by National Institutes of Health Research Grant GM-19398 from the National Institute of General Medical Sciences and by the Agricultural Experiment Station of the University of Minnesota.
