Ribosomal Changes during Induction of Cold Hardiness in Black Locust Seedlings

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ABSTRACT

Protein synthesis has been implicated in the cold-hardening process. Ribosomes from cold hardy and nonhardy black locust (Robinia pseudoacacia L.) seedlings were compared to determine if cold acclimation is related to alteration of ribosomal structure. Ribosomal structure, as indicated by thermal melting profiles, appears to be altered during induction of hardiness. Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins indicates at least 17 proteins from hardy seedlings that are different from those of nonhardy seedlings. These different proteins may be partially responsible for the different thermal melting profiles observed.

Plants capable of developing freezing resistance demonstrate altered metabolism during low temperature acclimation (cold hardening) (23). There have been many suggestions that protein synthesis plays a role in cold hardening (1, 4, 9, 13, 23). Among the many changes found during induction of cold hardiness are those which occur in RNA (13, 26) and protein (3, 4, 9, 13, 26) metabolism. This study was undertaken to examine the ribosome as related to induction of cold hardiness. The objective was to determine if ribosomal structure is altered during induction of hardiness.

MATERIALS AND METHODS

Plant Material. Black locust seedlings (Robinia pseudoacacia L.) were grown in heat-sterilized vermiculite. Moisture was maintained relatively constant. Two-month seedlings were cold hardened as outlined in Table I. At specific points in the cold-hardening regime, seedlings were placed into a programmed freezer and frozen at a rate of 6°C/hr. Samples were removed every 4°C starting at −4 and allowed to thaw overnight at 5°C. Stems were then harvested, placed into a mist chamber with a 15-hr photoperiod, allowed to recover for 7 days and visually evaluated for survival. Seedlings were considered unjured if their bark remained green and tightly bound to the stem. The bark of injured seedlings became brown and loose. Stems were assigned a value of 1 if uninjured, 0.5 if injured with 50% or more of the stem alive and 0 if dead or more than 50% injured. Values were totaled and divided by the total number of stems to determine percent survival (31).

Ribosome Isolation for Protein Extraction. Seedlings were harvested, stripped of leaves and rachises, cut into short sections and placed directly into liquid nitrogen. Sections were pulverized, using a Waring Blendor and previously chilled jar. The material remained frozen while being pulverized. The resulting powder was suspended in isolation buffer (50 mM tris-HCl, pH 8, 200 mM KCl, 25 mM MgCl₂) with 10% (w/v) sucrose and stored until warmed to 0°C. This suspension was squeezed through two layers of cheesecloth and centrifuged at 16,000 g for 20 min. The resulting supernatant was filtered through one layer of Miracloth and Triton X-100 was added to 0.1% (w/v) with a small amount of Antifoam A (Sigma). Approximately 50 ml of supernatant were layered over a 30 ml pad of 70% (w/v) sucrose in isolation buffer and centrifuged at 142,000 g at 4°C for 12 to 15 hr. The resulting pellet consisting of subunits, monosomes, polysomes, and residual debris was resuspended in isolation buffer and layered onto sucrose density gradients (20–50%, w/v) also prepared with isolation buffer, and centrifuged at 110,000 g at 4°C for 12 hr. Gradients were monitored at 254 nm. Subunits, monomers, and polysomes were collected, pooled, and precipitated for 1 hr at 0°C with an equivalent volume of cold ethanol (8). Ribosomes were pelleted at 15,000 g for 10 min, resuspended in a small quantity of supernatant and repelleted at 15,000 g for 10 min. The final pellet was stored overnight at −20°C or extracted immediately for ribosomal proteins.

Ribosomal Protein Extraction. Ribosomal protein was extracted by the 66% (v/v) acetic acid method of Waller and Harris (34) in the presence of 66 mM MgCl₂. Protein concentration was determined by the Lowry method (24), and the sample stored at −20°C until use for two-dimensional polyacrylamide gel electrophoresis.

Thermal Melting Analysis. Twenty to thirty stems were homogenized in 25 ml of Tm buffer (50 mM tris-HCl, pH 8, 50 mM KCl, 20 mM MgCl₂) with 10% (w/v) sucrose, squeezed through two layers of cheesecloth and centrifuged at 28,000 g at 4°C for 10 min. The supernatant was filtered through Miracloth and 3-ml aliquots were layered on top of sucrose density gradients (20–50%, w/v) also prepared with Tm buffer. Centrifugation and gradient analysis were performed as in the previous ribosome isolation. The central portions of the monosomal peaks were collected and retained in an ice bath until use. Thermal melting analysis was performed, using a Beckman DB-G spectrophotometer equipped with a Beckman Tm analyzer and a temperature bridge.

Electrophoresis of Ribosomal Proteins. The equipment and methods used were those suggested by Kaltenschmidt and Wittmann (19) with some minor modifications. Gel and electrophoretic buffer preparations are outlined in Table II. Protein solutions were thawed and a volume containing 2 to 4 mg of protein was freeze-dried. The protein then was suspended in sample gel solution, layered on top of a one-dimensional gel (16) (14.5 × 0.6 cm), and photopolymerized using a fluorescent desk.
Table I. Regime Used for Induction of Cold Hardiness in Black Locust Seedlings

<table>
<thead>
<tr>
<th>Duration</th>
<th>Day Length</th>
<th>Light Intensity(^1)</th>
<th>Temperature</th>
<th>Average Cold Hardness</th>
</tr>
</thead>
<tbody>
<tr>
<td>weeks</td>
<td>hr</td>
<td>μmol/m²/sec</td>
<td>Day</td>
<td>Night</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
<td>90</td>
<td>25</td>
<td>15</td>
</tr>
<tr>
<td>4</td>
<td>12</td>
<td>240</td>
<td>22</td>
<td>17</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>240</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>9</td>
<td>170</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^1\) Photosynthetically active radiation (400-700 nm).

Table II. Composition of Electrophoresis Gels and Electrode Buffers

<table>
<thead>
<tr>
<th></th>
<th>Sample Gel (pH 8.6)</th>
<th>Separation Gel (pH 8.6)</th>
<th>Electrode Buffer (pH 8.6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>First dimension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tris</td>
<td>80 mM</td>
<td>0.12 M</td>
<td>0.12 M</td>
</tr>
<tr>
<td>EDTA-Na₂</td>
<td>3.5 mM</td>
<td>6.5 MM</td>
<td>6.5 MM</td>
</tr>
<tr>
<td>Urea</td>
<td>8.0 M</td>
<td>6.0 M</td>
<td>6.0 M</td>
</tr>
<tr>
<td>Boric acid</td>
<td>to pH 8.6</td>
<td>0.15 M</td>
<td>0.15 M</td>
</tr>
<tr>
<td>Acrylamide</td>
<td>4% (w/v)</td>
<td>5% (w/v)</td>
<td></td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>0.125% (w/v)</td>
<td>0.13% (w/v)</td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>0.2% (v/v)</td>
<td>0.29% (v/v)</td>
<td></td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>0.0005% (w/v)</td>
<td>0.04% (w/v)</td>
<td></td>
</tr>
<tr>
<td>Riboflavin</td>
<td>0.0025% (w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Second dimension</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>6.0 M</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 N KOH</td>
<td>0.96% (v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>5.23% (v/v)</td>
<td>1.65% (v/v)</td>
<td></td>
</tr>
<tr>
<td>Acrylamide</td>
<td>18% (w/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bisacrylamide</td>
<td>0.5% (v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TEMED</td>
<td>0.58% (v/v)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium persulfate</td>
<td>0.33% (w/v)</td>
<td>15% (w/v)</td>
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</tr>
</tbody>
</table>

lamp. Proteins were subjected to electrophoresis for 24 hr at 4 C at 120 v and a current of 5 mamp/tube. Upon completion of the first dimensional separation the gels were removed from their tubes, and the pH was lowered by placing them in an open Petri dish in a desiccator along with several small beakers of concentrated HCl (2). This method required about 10 min to lower the pH as determined by methyl red indicator dye incorporated into an additional gel column. Gels were placed on top of the slab holder of the chilled, sealed two-dimensional apparatus and chilled two-dimensional gel mixture was poured under them and allowed to polymerize at 4 C. Proteins were subjected to electrophoresis for 45 hr at 100 v and a current of 100 to 120 mamp/slab.

Determination of Protein Migration. At the end of separations, gels were removed from holders, placed on stainless steel screens (where they remained for the entire staining-destaining process) and placed into 0.01% (w/v) Amido black in 10% (v/v) acetic acid overnight. They were rinsed briefly with distilled H₂O and placed into a continuously circulating 10% (v/v) acetic acid bath. The acid was cycled through an activated charcoal filter which removed excess dye dialyzing from the gels. Complete destaining took about 4 to 6 days. Gels were stored at 4 C in 10% (v/v) acetic acid until analyzed for protein locations.

Protein locations were determined by placing gels on a clear plastic grid (5 lines/2.54 cm) and recorded on graph paper of identical spacing. Although protein patterns were comparable between runs, the actual migration distances varied slightly. To facilitate comparison between separations, data had to be standardized. This was performed by using two proteins which always migrated to the same relative location in all separations as a reference point. The center of the reference proteins' spot was adjusted to a standard position and centers of all other proteins were adjusted to maintain the same relative distances from the reference proteins. Means, standard errors, and standard deviations were determined for proteins which appeared in at least 60% of either control or hardened separations. A schematic diagram (Fig. 1) was prepared by plotting the means. If the standard errors of a control and a treatment protein were overlapping they were plotted as one.

**RESULTS AND DISCUSSION**

Induction of Cold Hardiness. Cold hardiness in 2-month-old seedlings increased from -5 to -12 C during induction. The regime did not induce full dormancy; buds broke in three days...
on seedlings placed into the mist chamber at completion of the regime. The maximum degree of hardiness achieved for any one seedling was −20°C. Black locust in a natural environment has been observed to develop freezing resistance to at least −70°C (30).

Ribosome Extractions. Monosomes used for Tm analysis had 260–280 nm ratios close to 1.6, which indicated relatively little contamination. Clean ribosomes of Xenopus have a ratio of 1.85 (6), kidney bean 1.7 to 1.9 (7), and wheat leaves as high as 2 (17). Ribosomes used for protein extractions were not as clean. The 260–280 ratio was between 1.3 and 1.5, indicating the presence of some protein contamination. Crude ribosomal preparations may more closely represent the state of the ribosome in the cell (10). The concentration of K+ used in the isolation buffers decreases or prevents random adsorption of protein to ribosomes (33, 35). Assuming the ribosome contains 1,700,000 daltons of protein and is synthesizing a protein of 25,000 daltons, nascent protein would comprise less than 1.5% of the total protein of the ribosome (15). Unless a large percentage of all the ribosomes were at the same stage of synthesis of the same protein when isolated, nascent protein should not have been a serious contamination problem. Postmicrosomal supernatant proteins do not migrate with ribosomal proteins when fractionated together using two-dimensional polyacrylamide gel electrophoresis (7). Cold-hardened material appeared to yield nearly twice the concentration of ribosomes as did comparable quantities of control material. An increase in ribosome concentration during hardening has been reported previously (30).

Ribosomes of other species are heterogeneous with respect to their protein populations. Changes in the protein of the ribosome may be related to the functional state of the ribosome (22). If ribosomes of black locust also are heterogeneous, and if the majority of ribosomes in hardened material are in a different functional stage than those of controls, specific protein populations might differ. One method of avoiding this problem would be to convert all ribosomes to a functionally equivalent stage in the protein synthetic cycle prior to protein extraction. This would require isolation of active synthetic systems rather than just ribosomes, which would be difficult with bulk ribosome extractions.

Thermal Melting Analysis. Performance of Tm analysis at the ion concentrations used causes ribosomes to precipitate (21, 32). This discussion presumes that effects of precipitation would be equal for control and hardened material. Seven repetitions each of control and hardened ribosomes were performed. Figure 2 shows that the degree of hyperchromicity is greater for hardened ribosomes while the Tm point is greater for control ribosomes. Thermal melting profiles are an indication of secondary structure of rRNA in the ribosome while Tm points are an indication of structural stability. Guanine-cytosine pairs are more stable than AU pairs which causes rRNA with relatively more GC pairs to have higher Tm points. Adenine-uracil pairs contribute more to the increase in absorbency at 260 nm; thus, relatively more AU pairs should cause a greater degree of hyperchromicity (5). The Tm profiles obtained indicate changes in ribosomal structure of hardened seedlings. Control material may have a greater quantity of GC pairs and hardened material more AU pairs. This may indicate differences in base ratios of the primary structures. Drought stress leads to synthesis of rRNA with more GC pairs. Low temperature stress might have a similar effect (20). Ribosomal RNA base compositions during induction of hardiness have not been reported. Protein–rRNA associations may be different in hardened ribosomes, also causing different rRNA folding patterns and changing relative amounts of AU and GC pairs contributing to the total base pairing. Pea

![Fig. 2. Thermal melting profiles of ribosomes from control and cold-hardened black locust seedlings. Heating rate was 3.3 C/min. Control ribosomes (-----); cold-hardened ribosomes (••••).](image)

![Fig. 3. Effect of heating rate on thermal melting profiles of ribosomes from control black locust seedlings. 1.1 C/min (-----); 3.3 C/min (••••).](image)

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**Fig. 2.** Thermal melting profiles of ribosomes from control and cold-hardened black locust seedlings. Heating rate was 3.3 C/min. Control ribosomes (-----); cold-hardened ribosomes (••••).

**Fig. 3.** Effect of heating rate on thermal melting profiles of ribosomes from control black locust seedlings. 1.1 C/min (-----); 3.3 C/min (••••).

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**Abbreviations:** AU: adenine-uracil; GC: guanine-cytosine; TEMED: N,N,N',N'-tetramethylethylenediamine.
seed ribosomal proteins stabilize double-stranded regions of rRNA (12). Ribosomes from *Escherichia coli* and *Bacillus stearothermophilus* have different Tm profiles but contain rRNA with identical Tm profiles. The difference in ribosomal Tm's may be due to usual packing arrangements of ribosomal protein and rRNA, possibly because of differences in primary structures of the proteins (27).

RNase activity present on ribosomes contributes to increases in absorption observed in some ribosomal thermal melting analysis studies (28). If RNase were a factor in absorbency increases, a time factor would be involved. Thermal melting profiles were found to be related only to temperature in this study regardless of heating rates (Fig. 3).

**Protein Fractionation.** Five separate isolations of hardened ribosomes and six of control ribosomes were performed. Fractionation of basic proteins from these isolations produced seven control and eight treatment separations which were usable for analysis of protein location. Photographs of representative separations are shown in Figure 4. Fractionation of acidic proteins also was attempted but the results were too variable for conclusive analysis. Not all spots observed were necessarily different proteins since aggregation can cause additional spots in the region of high mol wt proteins (*i.e.* the top of the second dimension gel) (29). Varying amounts of protein remained at the origin or migrated only in the second dimension; however, other investigators have found no new proteins existing in these fractions (29, 36). The schematic representation (Fig. 1) of the separations shows 48 control and 38 hardened ribosomal proteins. Although there are more proteins in the ribosome, only those more repeatable in relative locations are represented in this diagram. Proteins which appear to migrate to the same location are not necessarily identical. The fractionation system employed separates proteins on the basis of charge in the first dimension and size in the second (29). It seems unlikely that two different proteins would have both the same charge and size. A change in only one amino acid can make a protein migrate to a different location with this system (18). Nineteen control and hardened proteins were found to have overlapping standard errors. The standard error of protein D also overlapped several hardened spots, but inspection of the separations indicated that C, B, and D appear either as one spot or as three very close spots. The difference may be due to slightly differing concentrations of proteins in different separations. Proteins 24 to 27 which appeared in the same area of hardened fractionations always were discernable as three or four separate spots as reflected by the distances between their means. Four hardened protein means are significantly different at the 5% level from all control proteins. The standard errors of another 12 do not overlap those of any control. Assuming there are about 80 ribosomal proteins, as in other plant species (11, 16, 25), these data suggest that at least 25% of the ribosomal proteins of cold-hardened black locust seedlings differ from those of nonhardy seedlings.

Presently the precise roles of the different ribosomal proteins of eucaryotes remain unknown. In light of reported relationships

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**Fig. 4.** Representative separations of ribosomes from black locust seedlings using two-dimensional polyacrylamide gel electrophoresis. The origins were the upper left-hand corners. Migration in the first dimension was from left to right; the second dimension was from top to bottom. A: Control ribosomal proteins; B: Cold-hardened ribosomal proteins.
between structure and function for ribosomes (22), the changes observed in this study may represent changes in ribosomal function. Protein synthetic mechanisms do appear to remain active during the winter (4). If this is true, the ribosome may be altered to function at lower temperatures. The cytoplasm of the cell is greatly altered during induction of hardiness (1, 23). Perhaps the ribosome must adapt to remain active in this altered cytoplasmic environment. Different proteins apparently play a role in cell hardening (14); the ribosome may be altered to produce different proteins to perform these roles. One important but unanswered question is whether a change in ribosomes leads to increased hardiness, or whether an increase in hardness alters ribosomes. This might be resolved by precisely establishing the time at which ribosomal structures become altered in relation to increasing hardiness of black locust seedlings.

LITERATURE CITED