The Effects of Salt on the Pattern of Protein Synthesis in Barley Roots

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ABSTRACT
The effect of salt stress on the incorporation of l-35S)methionine into protein was examined in roots of barley (Hordeum vulgare L. cv California Mariout 72). Plants were grown in nutrient solution with or without 200 millimolar NaCl. Roots of intact plants were labeled in vivo and proteins were extracted and analyzed by fluorography of two-dimensional gels. Although the protein patterns for control and salt-stressed plants were qualitatively similar, the net synthesis of a number of proteins was quantitatively changed. The most striking change was a significant increase in the relative amount of polypeptides with molecular weights of 6.3 and 6.5. Each pair consisted of proteins of approximately 26 and 27 kilodaltons (kD). In roots of control plants, the 27-kD proteins were more heavily labeled in the microsomal fraction relative to the 26-kD proteins, whereas the 26-kD proteins were enriched in the post 178,000 g supernatant fraction; in roots of salt treated plants, the 26- and 27-kD proteins were more intensely labeled in both fractions. Labeling of the 26- and 27-kD proteins returned to control levels when salt-stressed plants were transferred to nutrient solution without NaCl. No cross-reaction was detected between the antibody to the 26-kD protein from salt-adapted tobacco cells and the 26- and 27-kD proteins of barley.

Plants are often subjected to a wide variety of environmental stresses throughout their life cycles. One approach to understanding the ability of plants to tolerate environmental stresses is to identify stress-induced changes in the levels of individual proteins, with the assumption that adaptation to stress is the result of altered gene expression. Protein synthesis responds dramatically to environmental stresses such as heat shock (21, 28) and anaerobiosis (27) where the synthesis of most proteins ceases and the synthesis of a new set of proteins is induced. A similar response in the pattern of protein synthesis has been observed for excision shock (32). For other environmental stresses the response is not as dramatic; however, water stress (7, 18, 31), osmotic shock (19), wounding (28, 29, 30), cold acclimation (5, 17, 33, 34), and salt stress (12, 31) result in an increase in the net synthesis of some proteins and a decrease in the synthesis of others, with or without a concomitant induction of unique stress proteins.

We have initiated studies to analyze the influence of salt stress on the pattern of protein synthesis in barley roots. Barley is the most salt tolerant grain of major agricultural importance and has been grown in fields salted-out by previous irrigation practices (11, 25). Barley genetics and physiology have been extensively studied (4), as has the relationship between salt tolerance and ion transport both on a cellular and whole plant level (reviewed: 16, 20, 26). However, little work has been done on the effect of salt stress on protein synthesis in barley. Barley was included in studies of the effects of ions and organic solutes on the in vitro stability of polysomes (3) and the in vitro translation of mRNAs (15) of salt-tolerant and salt-sensitive plants.

Relatively few studies on the effect of salt stress on protein synthesis in vivo have been done. Considerable information has been obtained on cultured tobacco cells adapted to grow in medium containing high levels of NaCl (1, 31 and references therein). Ericson and Alfini (12) reported that two protein bands (26- and 32-kD) were much more abundant and one protein band (26-kD) was unique on SDS polyacrylamide gels of cells adapted to grow on NaCl. In more detailed studies, Singh et al. (31) found that eight protein bands increased, including the 26-kD polypeptide, and that four protein bands decreased in the salt-adapted cells; the 26-kD polypeptide increased in the greatest amount and constituted about 10% of the total cellular protein.

In this study we describe the effects of NaCl on in vivo protein synthesis in barley roots using 2D PAGE. The response of protein synthesis to salt stress is characterized with respect to NaCl concentration and length of treatment as well as to recovery from NaCl stress. Changes that occur in the synthesis of proteins found in cytosol and microsomal fractions are also described. Antibodies to the 26-kD protein of cultured tobacco cells were used to determine if the proteins of similar mol wt in barley were immunologically related to the tobacco protein.

MATERIALS AND METHODS
Plant Material. Seeds of barley (Hordeum vulgare L. cv California Mariout 72) were sown on moist cheesecloth supported by stainless steel mesh above 2 L of aerated nutrient solution. Plants were grown with 100% humidity at 22°C in the dark for 5 to 8 d. The nutrient solution was that described by Epstein and Norlyn (10) and was maintained at pH 5.6.

Salt Treatments. NaCl was added to the nutrient solution either when the seeds were sown (salt-grown plants) or 5 d after sowing for a period of 24 h (salt-shocked plants). For recovery experiments, the salt solution was replaced with fresh nutrient solution without salt for 24 to 48 h.

Plant Measurements. For each treatment, 5 g of barley seeds (approximately 100 seeds) were sown. Following treatments, length to the nearest mm and fresh weight to the nearest mg was determined for 25 plants. Percent germination was determined for the total number of seeds sown per treatment. For dry weight, 25 shoots or roots were placed in 5 ml shell vials and lyophilized overnight (Virtis Unitrap II); weight was measured.

1 Abbreviations: 2D, two-dimensional; NP-40, Nonidet P-40.
2 Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.
to the nearest 0.1 mg (Cahn Electrobalance, model 7500).

**In Vivo Labeling.** Intact plants were labeled to avoid wounding-induced changes in protein synthesis (8, 17, 29, 32) that could occur if excised root segments were used. Following treatments, single plants were placed in small vials with the roots immersed in 2 ml of the nutrient solution (the same solution the plants were treated in) containing 50 μCi of [35S]methionine (approximately 1100 Ci/mmole) or 4.1 × 10^13 Bq/mmol; New England Nuclear). Labeling was done for a period of 1 h at room temperature (approximately 22°C).

Measurement of Uptake and Incorporation of Label. Salt (200 mm) was added to the nutrient solution of 5 d old barley plants. After 0, 2, 4, 6, and 24 h, plants were labeled for 1 h as above, but with 10 μCi of [35S] methionine. The roots of labeled plants were washed with water, excised, blotted, and weighed. The roots were then ground in extraction buffer (see below). As a measure of uptake of [35S]methionine, aliquots of the homogenate were placed on glass microfiber filters (Whatman GF/A), dried, and counted in aqueous counting scintillant (ACS II, Amersham). The results obtained by this method were similar to those obtained when whole roots were counted and had the advantage that TCA precipitable counts could also be determined from the same sample. To quantitate incorporation of [35S]methionine into protein, aliquots of the homogenate were placed in 10% (w/v) TCA and incubated on ice for 30 min. The samples were filtered onto glass microfiber filters and the filters were washed three times with 5 ml of cold 5% (w/v) TCA and two times with 5 ml of 95% ethanol. After drying, the filters were counted using a Packard Tri-Carb 4430 scintillation counter.

Preparation of Cellular Fractions. Two barley plants were labeled with [35S]methionine as described above. The roots were washed with cold water, cut from the seed, and homogenized in 500 μl of 25 mm Tris plus 4 mm EDTA (pH 8.0) and 2 mm DTT in 0.25 m sucrose. The total homogenate was layered over 75 μl of 40% (w/v) sucrose containing 1 mm Tris-Cl (pH 7.2) and 1 mm DTT and centrifuged (178,000 g for 20 min in a Beckman Airfuge). Following centrifugation, the upper phase, including the material at the interface, was transferred to another tube, mixed, and recentlyrifuged. The pellet, containing microsomal membranes, and the supernatant, containing the cytosol, were prepared for gel electrophoresis.

Two-Dimensional Gel Electrophoresis. Proteins were extracted essentially by the method of Schuster and Davies (28). The roots of 10 intact plants were washed three times with 10 ml of cold water, cut from the seed, and ground in 500 μl of extraction buffer (0.7 M sucrose, 0.5 M Tris, 30 mM HCl, 50 mM EDTA, 0.1 M KCl, 2% [v/v] 2-mercaptoethanol) with a mortar and pestle. The sample was extracted immediately with phenol and the recovered protein solubilized in 100 μl of 9 M urea, 4% (v/v) NP-40, 2% (v/v) 2-mercaptoethanol, and 2% (v/v) ampholytes (9). The samples were incubated for 1 h at room temperature and insoluble material removed by centrifugation (178,000 g for 15 min in a Beckman Airfuge). Proteins from the microsomal and cytosolic fractions were phenol extracted and solubilized for electrophoresis as above except that an equal volume of 2x extraction buffer was added to the supernatant. Protein samples were stored at -70°C.

Two-dimensional PAGE was done as described previously (19); 200,000 cpm were loaded per gel. A carboxymethyl charge train, constructed with creatine phosphokinase by the method of Anderson and Hickman (1), was used for internal isoelectric point standardization of the 2D gels. Following electrophoresis of the second dimension, gels were processed for fluorography by the method described by Garrels (14). Kodak XAR-5 film was placed in contact with the dried gels and exposed at -70°C. The fluorographs photographed for the figures in this paper are representative of at least three experiments. Because the number of proteins undergoing quantitative changes was so large, only the most obvious and reproducible changes are indicated on the figures.

**Immunoblottine.** Protein samples were solubilized from barley roots and tobacco cells with a buffer containing 4% SDS by the method of Hurkman and Tanaka (19); however, the acetone precipitation step was omitted. Proteins were separated on SDS-polyacrylamide gels identical to those used for the second dimension of the 2D gel system except that the resolving gel was 13 cm and the stacking gel was 3 cm long. For the barley samples, 20 μg of protein was loaded per lane and for the tobacco cell samples, 10 μg of protein was loaded per lane. Protein determinations of samples precipitated from the SDS buffer (19) were made by the method of Lowry et al. (22). Proteins were transferred to nitrocellulose membranes (pore size 0.45 μm; Schleicher and Schuell) using a Trans-Blot Cell (Bio-Rad Laboratories). The gel was equilibrated for 20 min in transfer buffer (25 mm Tris, 192 mm glycine, 0.02% [w/v] SDS, 20% [v/v] methanol). Transfer was done for 18 to 20 h at 0.1 amp with tap water running through the cooling coil. Efficiency of transfer was verified by silver staining the blotted gel (24) and by staining a control blot with Aurolite (Janssen Life Sciences Products, Piscataway, NJ), a stain as sensitive for protein detection on nitrocellulose membranes as silver is for polyacrylamide gels. Immunodetection was done using the goat anti-rabbit, horseradish peroxidase Immunoblot kit from Bio-Rad. The primary antibody to the tobacco 26-kD protein and the S-0 (unadapted) and S-25 (adapted to grow on 25 g NaCl/L nutrient solution) tobacco cell samples were obtained from Dr. Ray Bressan, Purdue University.

**RESULTS**

Growth of Barley Seedlings in the Presence of NaCl. Barley seedlings were grown in the dark at 100% humidity in a standard nutrient solution in order to study salt-induced changes in a system where results would not be complicated by physiological processes such as photosynthesis and transpiration or by changes in light intensity and relative humidity. The effect of increasing concentrations of NaCl on germination, length, fresh weight, and dry weight of roots and shoots was measured to determine an appropriate concentration of NaCl to use for the protein labeling studies. Percent seed germination was not affected by NaCl concentrations up to 200 mm (Table I) and had a mean of approximately 96% for the four concentrations in this range. Germination was reduced by approximately 33% by 300 mm and 72% by 400 mm NaCl. Length and fresh weight of roots increased when plants were grown in 50 and 100 mm NaCl (Table I) and decreased with increasing NaCl concentrations above 50 mm. The dry weight of roots was similar for 0 to 100 mm NaCl.

Table 1. Effects of Salt on Roots of Barley

<table>
<thead>
<tr>
<th>mm NaCl</th>
<th>Length*</th>
<th>Weight</th>
<th>Dry Weightb</th>
<th>Germination</th>
</tr>
</thead>
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<tr>
<td></td>
<td>cm</td>
<td>mg</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>7.1 ± 0.8</td>
<td>63 ± 11</td>
<td>4.2 ± 0.7</td>
<td>6.7</td>
</tr>
<tr>
<td>50</td>
<td>9.2 ± 1.7</td>
<td>72 ± 14</td>
<td>4.3 ± 1.0</td>
<td>6.0</td>
</tr>
<tr>
<td>100</td>
<td>8.6 ± 1.1</td>
<td>66 ± 11</td>
<td>4.2 ± 0.8</td>
<td>6.4</td>
</tr>
<tr>
<td>200</td>
<td>6.1 ± 1.0</td>
<td>48 ± 8</td>
<td>3.1 ± 0.5</td>
<td>6.5</td>
</tr>
<tr>
<td>300</td>
<td>3.6 ± 0.8</td>
<td>34 ± 5</td>
<td>3.2 ± 0.5</td>
<td>9.4</td>
</tr>
<tr>
<td>400</td>
<td>1.1 ± 0.2</td>
<td>9.6*</td>
<td>1.4*</td>
<td>14.6</td>
</tr>
</tbody>
</table>

* The mean length of all roots per plant was first determined and then the mean and SE were calculated for the 25 plants. ** Dry weight)/ (fresh weight) × 100%. Average. For weighing accuracy, samples were combined.

Data are from a representative experiment.
PROTEIN CHANGES IN BARLEY ROOTS

mm NaCl, but decreased by approximately 25% in 200 and 300 mm NaCl and by approximately 67% in 400 mm NaCl. Dry weight, as a percent of fresh weight, was not affected by NaCl concentrations up to 200 mm and increased when plants were grown in 300 and 400 mm NaCl. Shoot length decreased with increasing NaCl concentration; no shoots were present on seeds sown over 400 mm NaCl (Table II). Fresh weight and dry weight of shoots decreased with increasing NaCl concentrations. Dry weight, as a percent of fresh weight, increased when plants were grown in 200 and 300 mm NaCl. The data in Tables I and II indicated that NaCl concentrations greater than 200 mm caused substantial changes in barley plants. Therefore, experiments were done using plants treated with 200 mm NaCl.

The effect of salt shock on plant growth was also examined; plants that were grown in nutrient solution without salt for 5 d were transferred to nutrient solution containing 200 mm NaCl for 24 h. Root elongation slowed; length and fresh weight were less than the corresponding controls while dry weight was the same (Table III). Shoot elongation was not significantly affected; length, fresh weight, and dry weight were all similar to the corresponding controls (Table IV). When the salt-shocked plants were transferred to nutrient solution without NaCl for a 24 h recovery period, the inhibition in root growth persisted (Table III), and shoot growth continued (Table IV). This pattern of inhibition of root growth and continued shoot growth lasted through an additional 24 h period (Tables III and IV).

Uptake and Incorporation of \[^{35}S\]methionine. In preliminary experiments, it was found that salt inhibited uptake and incorporation of \[^{35}S\]methionine into protein in roots of salt-shocked plants, but not in roots of salt-grown plants (Table V). Since approximately three times more counts were incorporated into

Table II. Effects of Salt on Shoots of Barley

Plants were 7 d old. The means and se are based on 25 plants per treatment. Data are from a representative experiment.

<table>
<thead>
<tr>
<th>mm NaCl</th>
<th>Length</th>
<th>Weight</th>
<th>Dry Weight*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cm</td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>0</td>
<td>12.6 ± 1.8</td>
<td>236 ± 38</td>
<td>14.0 ± 2.3</td>
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<tr>
<td>50</td>
<td>11.1 ± 1.2</td>
<td>191 ± 31</td>
<td>12.1 ± 2.4</td>
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<td>100</td>
<td>9.2 ± 0.9</td>
<td>162 ± 22</td>
<td>10.9 ± 1.8</td>
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<td>200</td>
<td>5.9 ± 1.0</td>
<td>86 ± 18</td>
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</tr>
<tr>
<td>300</td>
<td>2.3 ± 0.9</td>
<td>30 ± 12</td>
<td>3.7 ± 1.3</td>
</tr>
<tr>
<td>400</td>
<td>0*</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* (Dry weight)/(fresh weight) × 100%. * No shoots were present.

Table III. Effects of Salt Shock on Roots of Barley Plants

Plants were grown for 5 d in nutrient solution without NaCl and then 200 mm NaCl was added for 24 h (Shock). After treatment with NaCl, the plants were returned to nutrient solution without NaCl for 48 h (Recovery). Controls were untreated plants of the same age. The means and se are based on 25 plants per treatment. Data are from a representative experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age</th>
<th>Length*</th>
<th>Weight</th>
<th>Dry Weight*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>d*</td>
<td>cm</td>
<td>mg</td>
<td>%</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>6.1 ± 0.9</td>
<td>66 ± 12</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>Shock</td>
<td>6</td>
<td>5.4 ± 1.0</td>
<td>55 ± 11</td>
<td>4.4 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>7</td>
<td>7.4 ± 1.3</td>
<td>70 ± 16</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>24 h recovery</td>
<td>7</td>
<td>5.7 ± 0.9</td>
<td>55 ± 10</td>
<td>4.3 ± 0.7</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>7.6 ± 1.3</td>
<td>73 ± 22</td>
<td>4.3 ± 1.0</td>
</tr>
<tr>
<td>48 h recovery</td>
<td>8</td>
<td>5.6 ± 0.8</td>
<td>58 ± 15</td>
<td>4.0 ± 1.0</td>
</tr>
</tbody>
</table>

* Calculated as in Table I, footnote a. * (Dry weight)/(fresh weight) × 100%. * Days after sowing.

Table IV. Effects of Salt Shock on Shoots of Barley Plants

Plants were grown for 5 d in nutrient solution without NaCl and then 200 mm NaCl was added for 24 h (Shock). After treatment with NaCl, the plants were returned to nutrient solution without NaCl for 48 h (Recovery). Controls were untreated plants of the same age. The means and se are based on 25 plants per treatment. Data are from a representative experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Age</th>
<th>Length</th>
<th>Weight</th>
<th>Dry Weighta</th>
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<td></td>
<td>cm</td>
<td>mg</td>
<td>%</td>
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<tr>
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<td>10.3 ± 1.4</td>
<td>197 ± 29</td>
<td>11.9 ± 1.9</td>
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<tr>
<td>Shock</td>
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<td>10.3 ± 1.0</td>
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<tr>
<td>Control</td>
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<td>15.2 ± 2.4</td>
<td>257 ± 47</td>
<td>14.3 ± 3.0</td>
</tr>
<tr>
<td>24 h recovery</td>
<td>7</td>
<td>14.3 ± 1.9</td>
<td>231 ± 45</td>
<td>14.2 ± 3.0</td>
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<tr>
<td>Control</td>
<td>8</td>
<td>18.5 ± 2.6</td>
<td>293 ± 48</td>
<td>15.5 ± 3.4</td>
</tr>
<tr>
<td>48 h recovery</td>
<td>8</td>
<td>18.4 ± 1.6</td>
<td>249 ± 56</td>
<td>14.2 ± 3.4</td>
</tr>
</tbody>
</table>

a (Dry weight)/(fresh weight) × 100%. b Days after sowing.

Table V. Effect of 200 mm NaCl on Uptake and Incorporation of \[^{35}S\]methionine in Roots of Barley

Barley plants were labeled and cpm quantitated as described in "Materials and Methods." Data are from representative experiments.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Uptakea</th>
<th>Incorporationb</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10^-4/g fresh wt</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>339.5</td>
<td>35.7</td>
</tr>
<tr>
<td>Salt-grown</td>
<td>333.7</td>
<td>33.6</td>
</tr>
<tr>
<td>Salt-shocked</td>
<td>177.1</td>
<td>11.5</td>
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</table>

* cpm in total homogenate. b cpm in TCA-precipitable material.

FIG. 1. Changes in the uptake and incorporation of \[^{35}S\]methionine into roots of barley during salt shock. Following the addition of 200 mm NaCl, plants were labeled for 1 h at the times indicated. A, Comparison of uptake in control (C) and salt-shocked (■) plants; B, comparison of incorporation of label into TCA precipitable protein in control (C) and salt-shocked (■) plants.

TCA precipitable protein in control and salt-grown roots than in salt-shocked roots, a time course of the inhibition in salt-shocked roots was examined. Control and salt-shocked plants were removed at several time points during the 24 h treatment period and labeled for 1 h (Fig. 1). In the control roots, uptake increased as a function of time during the 24 h experimental period (Fig.
proteins labeled in vivo with [35S]methionine were separated on 2D gels. Fluorographs revealed that at least 650 polypeptides were separated by the 2D gel system. The newly synthesized proteins ranged in mol wt from approximately 18- to 120-kD and had pIs ranging from 4.5 to 7.5. The ampholyte range of 4.5 to 7.5 permitted the separation of the maximum number of proteins, although a number of proteins were also located at the acidic and basic regions of the 2D gels. Treatment of plants with NaCl did not induce striking qualitative differences in the pattern of protein synthesis. The protein patterns were similar for fluorographs of proteins labeled in vivo by control roots, by roots of salt-grown plants, and by salt-shocked plants (Fig. 2). Salt-induced changes in protein pIs were not found when the same samples were compared on silver stained gels that also contained the carbamylation charge train of creatine phosphokinase (not shown). NaCl did alter the relative amounts of a number of the proteins labeled in vivo. A comparison of the fluorographs of proteins from control roots (Fig. 2A) and roots of salt-grown plants (Fig. 2B) showed that growth in NaCl resulted in a reduction in the labeling of a number of proteins (approximately 75) and an increase in the labeling of others (approximately 50). These quantitative changes occurred in a heterogeneous group of proteins having a wide range of mol wt and pIs. The most prominent changes are indicated in Figure 2B. A comparison of the fluorographs of proteins from control roots and roots of salt-shocked plants (Fig. 2C) also showed that NaCl caused a reduction (approximately 75) or increase (approximately 25) in the labeling of a range of proteins and did not induce the synthesis of major new proteins. The most prominent protein changes are indicated in Figure 2C. It is noteworthy that many of the changes observed in salt-shocked roots were similar to those observed in salt-grown roots.

The most striking changes in labeling in roots of salt-grown or salt-shocked plants were significant increases in two protein pairs that had pIs of approximately 6.3 and 6.5 (indicated by arrows in Fig. 2). Each pair consisted of proteins of approximately 26- and 27-kD. In fluorographs of proteins from control roots (Fig. 2A), the pl 6.5 proteins were greater in amount than the pl 6.3 proteins. The 27-kD proteins in each pair were lower in amount than the corresponding 26-kD proteins. In fluorographs of proteins from roots of salt-grown plants (Fig. 2B), both the 26- and 27-kD proteins were labeled more intensely than in control roots and the pl 6.5 protein pair more than the pl 6.5 protein pair. Similar results were obtained in roots of salt-shocked plants (Fig. 2C), although the 26- and 27-kD proteins appeared to be labeled in relatively lower amounts compared to salt-grown plants.

To determine if the effects of NaCl on the pattern of protein synthesis were reversible, salt-grown plants were transferred to nutrient solution without NaCl for 48 h. A comparison of proteins labeled at the end of the 48 h recovery period (Fig. 2D) with those labeled by the control (Fig. 2A) revealed that nearly all of the proteins that had increased or decreased with NaCl treatment were labeled at control levels. The amounts of the 26- and 27-kD proteins decreased during the recovery period. The decrease in the labeling intensity of these proteins occurred within 24 h (data not shown) and showed no further decline after 48 h (Fig. 2D).

To test the effect of NaCl concentration on protein synthesis, plants were grown in several NaCl concentrations. Overall, the quantitative changes in protein labeling observed for plants grown in 100 and 300 mM NaCl were similar to those already described for the salt-grown (200 mM) plants; changes were less striking for plants grown in 50 mM NaCl (data not shown). Fluorographs of proteins from roots grown in 50 mM NaCl revealed a small increase in intensity of the pl 6.3 and 6.5 proteins, whereas a greater increase occurred in roots grown in 100, 200, and 300 mM NaCl (data not shown).

Two-Dimensional Gel Electrophoresis of Cytosolic and Microsomal Proteins. Cytosolic and microsomal fractions were prepared from roots of control and salt-grown plants that were labeled in vivo with [35S]methionine. Labeled proteins from each fraction were separated on 2D gels. A comparison of the fluorographs of 2D gels of the cytosolic (Fig. 3A) and microsomal (Fig. 3C) fractions showed that the protein patterns were qualitatively different. Overall, the majority of the cytosolic proteins were located in the acidic region (pI < 6.0) of the 2D gels (Fig. 3A) whereas comparatively more of the microsomal proteins were located in the basic region (Fig. 3C). Each of the fractions contained approximately 40 proteins that were enhanced in or specific to that fraction; prominent proteins characteristic of the cytosolic and microsomal fractions are indicated (diamonds) in Fig. 3, A and C. As in fluorographs of total proteins from roots, NaCl caused quantitative but not qualitative changes in the labeling pattern of proteins of the cytosolic (Fig. 3B) and microsomal fractions (Fig. 3D). In general, more quantitative changes were evident in fluorographs of proteins from the cytosolic fraction of salt-treated roots than in the microsomal fraction. In the cytosolic fraction, five proteins with mol wt of approximately 35- to 45-kD (indicated by small arrows in Fig. 3B) increased significantly in addition to the 26- and 27-kD proteins.

Although many proteins were restricted to either the cytosolic or microsomal fraction, the 26- and 27-kD proteins were present in both fractions; the pl 6.5 proteins were present in greater amounts relative to the pl 6.3 proteins (Fig. 3, A and C). However, the 26-kD proteins were much more abundant relative to the 27-kD proteins in the cytosolic fraction (Fig. 3A). Fluorographs of proteins synthesized in roots of salt-grown plants revealed an increase in the amounts of the 26- and 27-kD proteins in both fractions (Fig. 3, B and D). In the cytosolic fraction, the 26-kD proteins were present in much greater amounts relative to the 27-kD proteins (Fig. 3B). In the microsomal fraction, the 26- and 27-kD proteins were all present at increased levels (Fig. 3D); the protein pair at pl 6.3 was present at slightly higher levels relative to the pl 6.5 proteins.

Immunoblots. To determine if the 26- and 27-kD proteins of barley were related to the 26-kD protein of salt-adapted tobacco cells, a SDS-polyacrylamide gel of proteins solubilized from roots of control and salt-grown barley plants and from S-0 (unadapted) and S-25 (adapted) tobacco cells was blotted to nitrocellulose. The blot was treated with the antibody to the tobacco 26-kD protein. The antibody cross-reacted strongly with proteins solubilized from the tobacco cell lines (Fig. 4). The cross-reaction was primarily with one protein band that, in our gel system, had an apparent mol wt of 24,000. The cross-reaction product was greater for the S-25 cells than the S-0 cells. The antibody did not cross-react with the 26- and 27-kD proteins of barley roots. A blot stained with AurolDye showed that the majority of proteins,
Fig. 2. Fluorographs of $^{35}$S-labeled proteins of barley roots resolved by 2D PAGE. Plants were grown with or without 200 mM NaCl and proteins labeled in vivo with $[^{35}S]$methionine. A, Plants grown in nutrient solution without NaCl (control); B, plants grown in nutrient solution plus 200 mM NaCl for 6 d (salt-grown); C, plants grown in nutrient solution for 5 d and 200 mM NaCl added for a period of 24 h (salt-shocked); D, plants grown as in (B) and transferred to nutrient solution without NaCl for 48 h (recovery). (○), Proteins that increase and (□), proteins that decrease with respect to the control (A). Arrows indicate the pl 6.3 and 6.5 protein pairs that increase significantly with NaCl stress.
Fig. 3. Fluorographs of 35S-labeled proteins of cytosolic and microsomal fractions of roots of barley resolved by 2D PAGE. Plants were grown with or without 200 mM NaCl and proteins labeled in vivo with [35S]methionine. A, Cytosolic proteins of roots of plants grown in nutrient solution without NaCl (control); B, cytosolic proteins of roots of plants grown in nutrient solution plus 200 mM NaCl for 6 d (salt-grown); C, microsomal proteins of roots of plants grown in nutrient solution without NaCl (control); D, microsomal proteins of roots of plants grown for 6 d in nutrient solution plus 200 mM NaCl (salt-grown). (○), Proteins that are enhanced in or specific to the cytosolic or microsomal fraction; large arrows indicate the pI 6.3 and 6.5 protein pairs that increase significantly in the cytosolic fraction with NaCl stress; small arrows indicate proteins that increase significantly in the cytosolic fraction with NaCl stress; (□), proteins that increase and (□), proteins that decrease with NaCl stress.
PROTEIN CHANGES IN BARLEY ROOTS

including the 26- and 27-kD proteins of barley were efficiently transferred to the nitrocellulose (not shown). The tobacco antibody did cross-react nonspecifically with a few barley proteins (a control blot showed this was not due to binding of the goat antirabbit antibody to the barley proteins; data not shown).

DISCUSSION

Salt stress inhibits growth and alters protein synthesis in barley seedlings. Germination decreased substantially when seeds were sown in NaCl concentrations above 200 mM. All of the NaCl concentrations tested inhibited shoot growth, but root growth was stimulated by 50 and 100 mM NaCl. Salt shock also caused a significant decrease in growth of roots but not of shoots. Because NaCl concentrations greater than 200 mM caused substantial decreases in germination and growth, we used this concentration to study salt-induced changes in the incorporation of [35S]methionine into protein. The effect of NaCl on incorporation of [35S]methionine into the proteins of barley roots was complex. Our observations suggest that the response to NaCl does not involve the synthesis of unique proteins, but, rather, involves the modulation of the net synthesis of a wide range of constitutive proteins. Of the numerous changes, the amounts of two protein pairs with pIs of 6.3 and 6.5 and mol wt of 26,000 and 27,000 increased significantly in response to NaCl treatments. The labeling of the 26- and 27-kD proteins increased when plants were grown continuously in nutrient solutions supplemented with 50 to 300 mM NaCl or when plants were shocked by the addition of 200 mM NaCl to the control nutrient solutions. When plants, whether grown in NaCl or shocked with NaCl, were placed in nutrient solution without NaCl, the levels of nearly all proteins that had increased or decreased during NaCl treatment, including the 26- and 27-kD proteins, returned to near control levels.

In preliminary cell fractionation studies, it was found that NaCl altered the labeling of a number of proteins specific to or enhanced in the cytosolic and microsomal fractions. In roots of control plants, the 26-kD proteins were predominant in the cytosolic fraction whereas both the 26- and 27-kD proteins were present in the microsomal fraction. In roots of salt-treated plants, the 26- and 27-kD proteins increased in both the cytosolic and microsomal fractions. Although more salt-induced protein changes were evident in the cytosolic fraction, the fact that changes occurred in the microsomal fraction is interesting since membrane proteins may play an important role in the response of plants to salt stress. Under conditions of salt stress, high cytoplasmic Na+:K+ ratios cause metabolic damage and high Na+ concentrations may interfere with K+ acquisition (23 and references therein). Barley responds to salt stress by maintaining favorable cytoplasmic Na+:K+ uptake selectivity, by Na+ extrusion, and by vacuolar Na+ compartmentation (23 and references therein), functions that are all regulated by membranes (20). Lynch and Läuchli (23) speculated that part of the inhibition in stelar K+ release caused by salinization of barley roots may be due to the inhibition of synthesis or turnover of protein(s). We have shown that changes in the net synthesis of microsomal membrane proteins of barley roots do occur in response to salt stress. Other examples of quantitative alterations in the synthesis of membrane proteins in response to environmental stress include the protein changes that occur in the plasma membrane of winter rye (33) and mulberry (34) during cold acclimation; evidence indicates that the plasma membrane is intimately involved in cold acclimation and freezing injury (34 and references therein).

In cultured tobacco cells adapted to grow in media containing high levels of salt, a 26-kD polypeptide increases significantly (12, 31). This polypeptide constitutes up to 10% of the total cellular protein in adapted cells and is synthesized in two different periods during culture growth (31). The 26-kD polypeptide synthesized during the early growth phase has a pI of 8.2 and the one synthesized during the late growth phase has a pI of 7.8 (31). In addition, the 26-kD polypeptide is synthesized in unadapted cells and has similar partial proteolysis peptide maps and is immunologically cross-reactive with the polypeptide from adapted cells. The principle salt-induced proteins of barley roots resembled the tobacco proteins in that they had nominal mol wt of 26,000. However, the barley proteins were not immunologically cross-reactive with antibodies to the tobacco protein. The lack of cross-reactivity was not surprising because the pIs of the barley proteins were more acidic than those of the tobacco proteins and further, the barley proteins were 2- to 3-kD larger than the tobacco protein, which had an apparent mol wt of 24,000 on gels run in our electrophoresis system.

One explanation for the observed changes in net protein synthesis induced by NaCl may be that the translation of the mRNAs is inhibited or stimulated to varying degrees by increased cytoplasmic NaCl concentrations. Evidence from studies on the effects of ions (K+, Na+, Cl−) and compatible organic solutes (proline, glycinebetaine) on the in vitro translation of mRNAs isolated from salt tolerant and salt sensitive plants (15) showed that protein synthesis was inhibited by substitution of Na+ for K+ or of Cl− for acetate at concentrations above 80 mM (15). In addition, the relative synthesis of some polypeptides changed when Na+ or NH4+ were partially substituted for K+; these polypeptides were found to be synthesized by plastid RNA. Since

FIG. 4. Immunoblot of a SDS gel of proteins solubilized from barley roots and cultured tobacco cells. The blot was incubated with antibody prepared from the 26-kD protein of salt-adapted tobacco cells; cross-reactivity was detected by using the goat antirabbit, horseradish peroxidase assay. Lane A, proteins from roots of barley grown in nutrient solution without NaCl; lane B, proteins from roots of barley grown in nutrient solution with 200 mM NaCl for 6 d; lane C, proteins from S-0 (unadapted) cultured tobacco cells; lane D, proteins from S-25 (adapted) cultured tobacco cells.
chloramphenicol, an inhibitor of mitochondrial and plastid protein synthesis, was included in our labeling mixture, the changes in net protein synthesis caused by NaCl represent changes in translation of cytoplasmic mRNAs. Whether the alteration in the pattern of net protein synthesis induced by NaCl is due to changes in the efficiency of mRNA translation (initiation and elongation rates, amount of mRNA associated with ribosomes or polysomes, whether mRNA is in an active or inactive state) or due to regulation of mRNA transcription, processing, transport, or stability or due to altered rates of protein degradation cannot be deduced from these studies.

In this paper, we have demonstrated that the synthesis of proteins with mol wt of 26,000 and 27,000 increases significantly in barley roots grown in NaCl. Because the synthesis of these proteins decreases when plants are transferred to nutrient solution without NaCl, it is tempting to speculate that expression of the genes for these proteins may be involved in the ability of barley to tolerate salt. However, the 26- and 27-kD proteins may represent shock-induced proteins rather than proteins that allow barley to grow or survive better in the presence of salt. In this respect, Singh et al. (31) found, when salt-adapted tobacco cells were shocked by transfer to medium containing higher or lower levels of NaCl, that none of the newly synthesized proteins corresponded to the major polypeptides associated with salt adaptation. This finding would suggest that there may be differences between expression of genes induced by shock and where expression is altered only after adaptation. A better understanding of the role that altered gene expression plays in salt tolerance can be achieved when the genes coding for the salt-induced polypeptides are identified. The characterization of these genes will provide an opportunity to more accurately assess expression during salt stress as well as contribute to understanding the function of these polypeptides through their deduced amino acid sequences.

Acknowledgment—We thank Dr. Ray Bressan for generously providing us with S-0 and S-25 cultured tobacco cells and the antibody to the 26-kD protein.

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