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Salinity Stress Induced Tissue-Specific Proteins in Barley Seedlings

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ABSTRACT

Protein changes induced by salinity stress were investigated in two barley cultivars, California Mariout, a salt-tolerant variety and Prato, a salt-sensitive variety. Rapidly growing young barley seedlings were exposed to NaCl and the newly synthesized proteins were resolved on two dimensional polyacrylamide gels following isoelectric focusing or nonequilibrium pH gradient gel electrophoresis in the first dimension. Salinity induces distinct protein changes in root and shoot tissues. In roots, the salinity effects are identical in both cultivars. First, salinity modulates the synthesis of two different sets of proteins, one of which is elevated, and the other, depressed. Second, six new proteins are induced all of which are low in molecular weight, 24 to 27 kilodaltons, with an isoelectric point range of 6.1 to 7.6. In contrast to roots, salinity induces cultivar-specific shoot proteins. Five new shoot proteins are induced whose molecular weights and isoelectric points fall within the range of 20 to 24 kilodaltons and 6.3 to 7.2, respectively. Three of the newly induced proteins are unique to Prato. In addition, salinity inhibits the synthesis of a majority of shoot proteins. The new proteins produced in roots and shoots are unique to each tissue and their induction is apparently regulated coordinately during salinity stress.

The molecular basis of salinity tolerance among various plant species is not understood. Genotypic variation for salinity tolerance has been observed in many plants including barley (1, 15, 16). Barley cultivars differing in responses to salinity have been identified (4). An understanding of the molecular differences between such barley genotypes when exposed to salinity stress would be helpful.

In the present study, protein synthesis in two barley genotypes, California Mariout (CM72) and Prato, was investigated. Previous studies have indicated that the former variety is salt tolerant and the latter salt sensitive (4, 13). The results demonstrate that in rapidly growing young barley seedlings salinity stress induces specific protein changes in roots and shoots of the two genotypes.

MATERIALS AND METHODS

Plant Material and Salinity Treatment. Seeds of barley (*Hordeum vulgare*, L. cv CM72 and Prato) were germinated in 10 cm Petri plates at 27°C in 0.1 mM $CaSO_4$ under sterile conditions for 70 to 75 h. Seedlings were selected for uniform growth in a laminar flow hood and 1.5 to 2 cm of the apical parts of roots were dipped in a treatment solution (0.1 mM $CaSO_4$ ± NaCl) for approximately 2 min. Then, five seedlings were placed on each treatment plate such that root contact with the solution was insured and incubation continued in dark at 27°C for the times specified in text. This method of seedling transfer to treatment plates did not alter the growth rate compared to nontransferred seedlings.

***In Vivo* Labeling of Proteins with ^{35}S-methionine.** Root and shoot tissues were sampled from the control and NaCl-treated seedlings at various times for labeling with ^{35}S-methionine. Thirty root tips representing 0.6 to 0.8 cm of the apical regions were agitated at 150 rpm with 50 μCi of ^{35}S-methionine (1120 Ci/mmol, New England Nuclear) in 0.5 ml of the corresponding treatment solution for 2 to 3 h at 25°C. Five shoots (primary leaf with coleoptile) were excised at the nodal region separating the radicle and plumule. The first 1.5 cm from the node was isolated, halved, and transferred to 1 ml of a labeling solution (50 μCi ^{35}S-methionine) and incubated as above for roots. All excisions were made under liquid and all operations conducted under sterile conditions. After labeling, the tissues were harvested on two layers of Miracloth by filtration, washed with cold water, and frozen in liquid N_2. Salinity induced protein changes described in "Results" did not result from tissue excision per se as similar patterns were also obtained by labeling intact tissues.

Extraction and Preparation of Proteins for 2-D Gels. Proteins were prepared by slight modifications of a method of Shuster and Davies (17). The frozen tissue was ground under liquid nitrogen in a mortar and suspended in 2.5 ml of an extraction buffer (0.5 M Tris-HCl, pH 7.5, 0.1 M KCl, 0.05 M Na_2EDTA, pH 7.4, 2% 2-mercaptoethanol, and 0.7 M sucrose). Immediately, an equal volume of redistilled phenol saturated with water was added to the cell extract and the mixture shaken at room temperature for 10 to 15 min at 300 rpm on a New Brunswick G2 shaker. The phenol phase (upper layer) was collected by centrifugation at 10,000 rpm for 10 min in a Sorvall SS34 rotor or HB4 rotor, mixed with an equal volume of extraction buffer, shaken for 5 min and centrifuged. Five volumes of methanol containing 0.1 M ammonium acetate were added to the reextracted phenol phase and the proteins precipitated overnight at −20°C. The protein precipitate was collected by centrifugation and washed with methanol, NH_4Ac solution (3 × 5 ml) and once with −20°C acetone (5 ml). The proteins were air dried for 10 min at room temperature, resuspended in the O'Farrell (10) lysis buffer (9.5 M urea, 2% Nonidet P40, 2% ampholine pH 3.5 to 10 [LKB] and 5% 2-mercaptoethanol) and clarified in an Eppendorf microcentrifuge for 5 min at top speed. The samples were stored at −20°C (if used within 2 weeks) or at −76°C.

In the initial experiments, proteins prepared by the above method were compared to those prepared by a modified nuclease method (6, 10). In the latter procedure, the frozen tissue (100–200 mg fresh weight) was ground in liquid N_2, resuspended in 20 mM Tris-HCl (pH 7.5), 50 mM KCl, 5 mM $MgCl_2$, 1% 2-mercaptoethanol, and 0.3% SDS, boiled for 2.5 min, and re-

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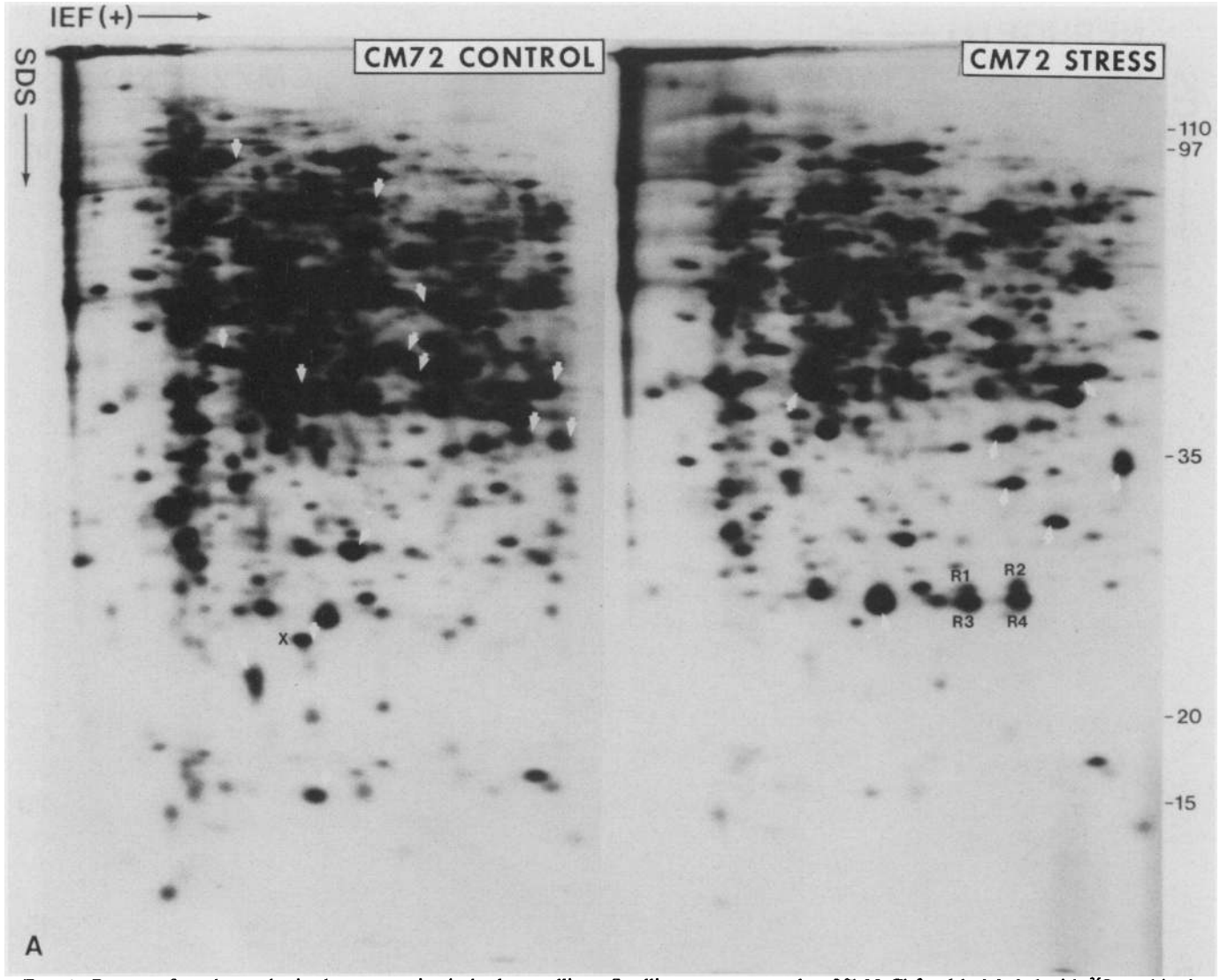

FIG. 1. Pattern of newly synthesized root proteins in barley seedlings. Seedlings were exposed to 2% NaCl for 6 h, labeled with ^{35}S-methionine, and proteins prepared. Proteins resolved by IEF/2-D and NEPHGE/2-D are shown in panels A and B, respectively. Only those proteins (basic) which are not displayed in panel A are shown in panel B. pH ranges (left to right) are 4 to 7 and 7 to 9.2 in panels A and B, respectively. Proteins of control and NaCl-treated (stress) seedlings are shown. Proteins whose synthesis is reduced on salinity stress are denoted by downward arrows in control panels (A and B). Proteins whose synthesis is enhanced on salinity stress are indicated by upward arrows in stress panels (A and B). R1 to R6 are new proteins induced by stress. A protein marked X in control panel A is found only in CM72 but not in Prato. Figure shows data from CM72. Control and salinity induced protein patterns in Prato were identical to those in CM72 and are not shown. Molecular weights are shown in kilodaltons.

Table I. *Uptake of ^{35}S-Methionine and Protein Synthesis during Salinity Stress in Roots and Shoots of Barley Seedlings*

Seedlings were treated with 2% NaCl for 6 h, tissues excised and incubated with ^{35}S-methionine for 2.5 h. Total tissue uptake of ^{35}S-methionine and its incorporation into TCA-insoluble proteins were determined as described (12). Incorporation into 30 roots or 5 shoots are shown. Values are means of two experiments.

Tissue	Treatment	Uptake		Incorporation[a]	
		CM72	Prato	CM72	Prato
		cpm × 10^{-6}			
Root	Control	40.4 (100)[b]	22.91 (100)	10.30 (100)	12.33 (100)
	NaCl	9.60 (24)	13.60 (59)	3.81 (37)	2.70 (22)
				15.90 (154)[c]	4.60 (37)[c]
Shoot	Control	2.23 (100)	1.12 (100)	1.86 (100)	2.67 (100)
	NaCl	0.44 (20)	0.27 (24)	0.25 (13)	0.37 (14)
				1.25 (67)[c]	1.54 (58)[c]

[a] Values corrected for uptake differences between the two genotypes. [b] Numbers in parentheses are percentages. [c] Corrected for uptake differences caused by salinity treatment in addition to genotype.

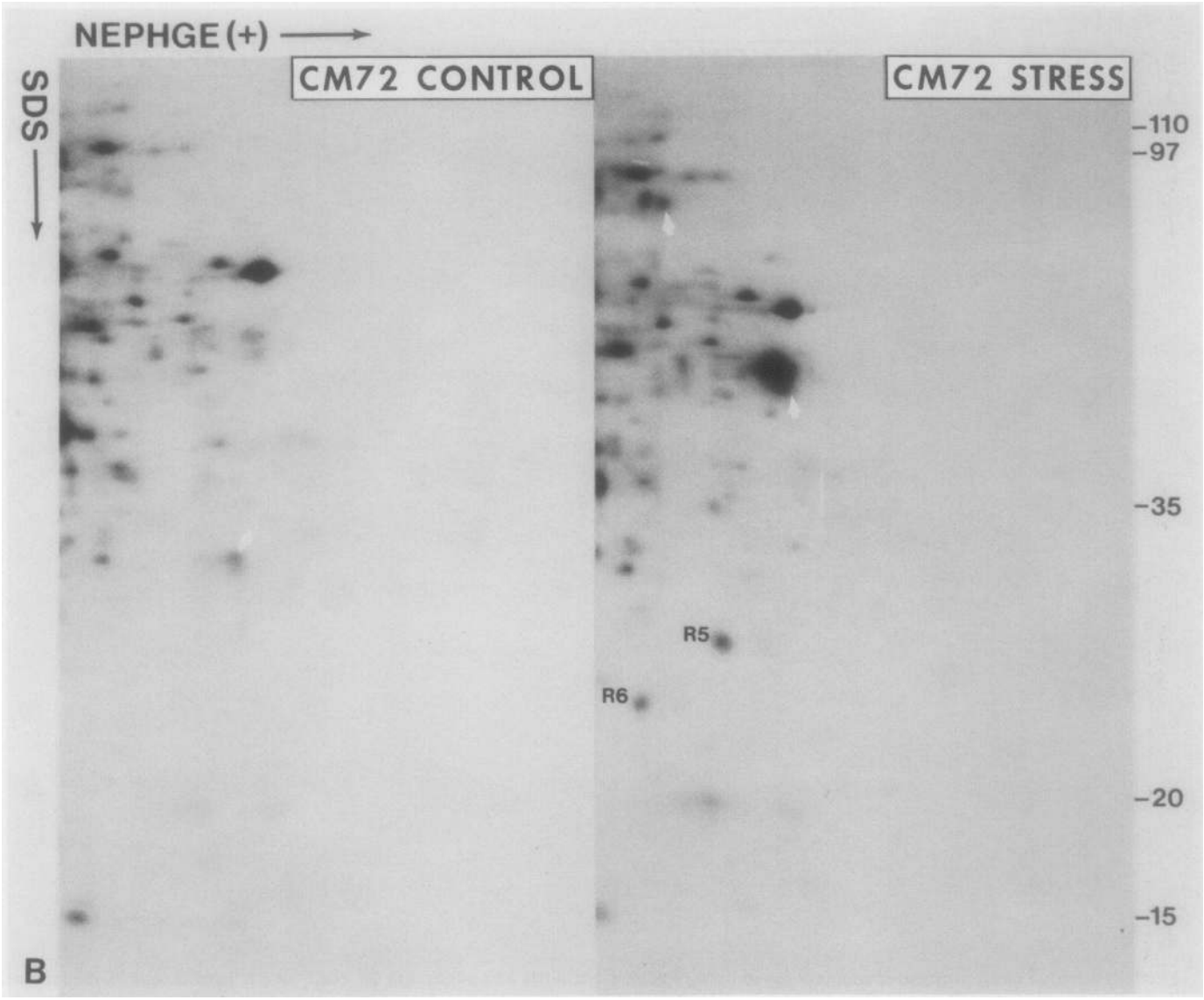

FIG. 1B.

Table II. *Properties of New Proteins Induced by Salinity Stress in Barley Seedlings*

Tissue	Protein	Method[a]	$M_r \times 10^3$	pI	CM72	Prato
Root	R1	A	27.2	6.1	+	+
	R2	A	27.1	6.3	+	+
	R3	A	26.0	6.1	+	+
	R4	A	26.5	6.3	+	+
	R5	B	26.2	7.6	+	+
	R6	B	24.5	7.0	+	+
Shoot	S1	A	21.0	6.3	−	+
	S2	A	20.3	6.4	−	+
	S3	B	24.0	7.2	+	+
	S4	B/A	20.5	6.8	+	+
	S5	B	20.0	7.0	−	+

[a] Method A, IEF/2-D; B, NEPHGE/2-D; + and − refer to presence or absence of a protein in the specific cultivar. Data are means of two determinations.

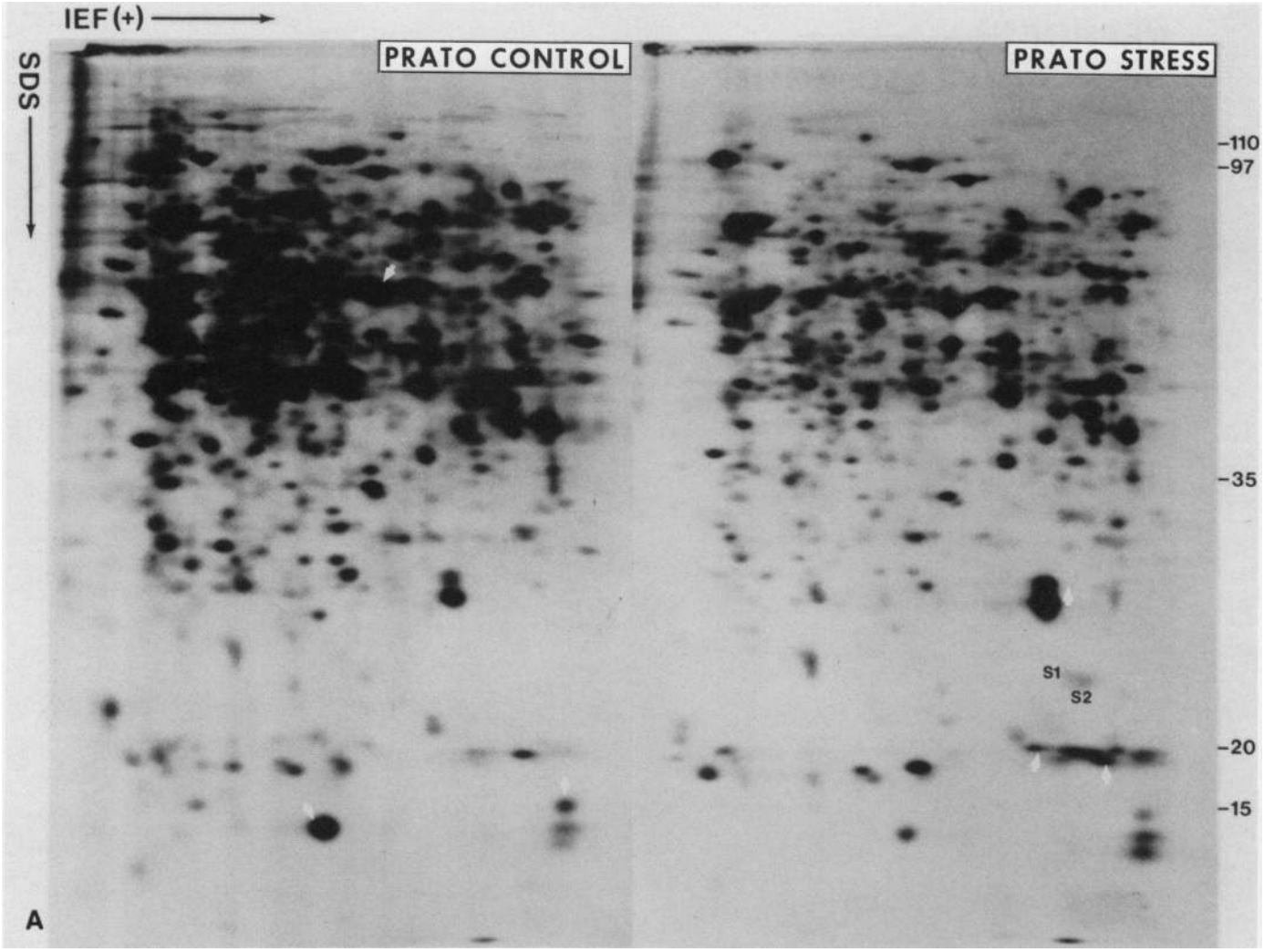

FIG. 2. Pattern of newly synthesized shoot proteins in barley seedlings (Prato). Details are same as described in Figure 1. Panel A is IEF/2-D; panel B, NEPHGE/2-D. S1 to S5 are new shoot proteins induced by stress.

turned to ice. Ten μl of a nuclease solution (1 mg/ml each of RNase A and DNase 1) were added, mixed, and left on ice for 15 min. Then, the tissue extract was lyophilized, suspended in the O'Farrell lysis buffer, and clarified as in the phenol method.

Both methods gave comparable results on 2-D gels, and only the phenol method was employed in all experiments presented.

Electrophoresis of Proteins on 2-D Gels. Proteins were resolved by IEF[2] or NEPHGE in the first dimension followed by SDS-PAGE in the second dimension. The methods of O'Farrell (10, 11) were used with slight modifications. The IEF gel contained 1.2% and 0.8% of pH 3.5 to 10 ampholine and pH 5 to 7 ampholine, respectively. After preelectrophoresis, the protein sample was applied at the acid end of the gel and electrophoresed at 400 V for 18 h. For NEPHGE, the first dimension gel was made with 2% pH 3.5 to 10 ampholine and there was no preequilibration electrophoresis. The proteins were applied as in IEF and electrophoresed for 4.5 h at 400 V. Each gel was equilibrated in 5 ml of 62.5 mM Tris HCl (pH 6.8), 2.3% SDS, 10% glycerol, and 50 mM dithiothreitol for 30 min with gentle agitation. The second dimension was a 12.5% SDS-polyacrylamide gel with a 4.75% stacking gel and done according to Laemmli (8). The pH gradient was linear from 4 to 7 and 4 to 9.2 in the IEF and NEPHGE methods, respectively. We estimate that three-fourths of the barley proteins are resolved in IEF gels and the remainder by NEPHGE.

Visualization of proteins was by silver staining (9) or by fluorography. For the former, equal amount of proteins was applied to the gel. For the latter, whenever possible, equal amount of radioactivity (400,000 cpm), based on trichloroacetic acid (TCA) precipitable counts of protein samples, was loaded on to gels. The proteins were fixed in 10% acetic acid, 50% methanol, gels treated with En^{3}hance (New England Nuclear), dried and exposed to Kodak XAR-5 film with a DuPont intensifying screen. When the amount of radioactivity applied to the gel was low, the x-ray film was exposed proportionately longer. All fluorographs were developed after exposing the films to an equivalent of 10^6 cpm per day.

RESULTS

In preliminary experiments, barley seedlings were treated for various periods up to 24 h with 0, 1, 2, and 3% NaCl and protein synthesis was assessed by SDS-PAGE in single dimension gels. A distinct change in protein pattern was apparent at the 2 and 3% NaCl levels after 4 h of exposure and no further changes

[2] Abbreviations: IEF, isoelectric focusing; NEPHGE, nonequilibrium pH gradient gel electrophoresis; 2-D, two-dimensional.

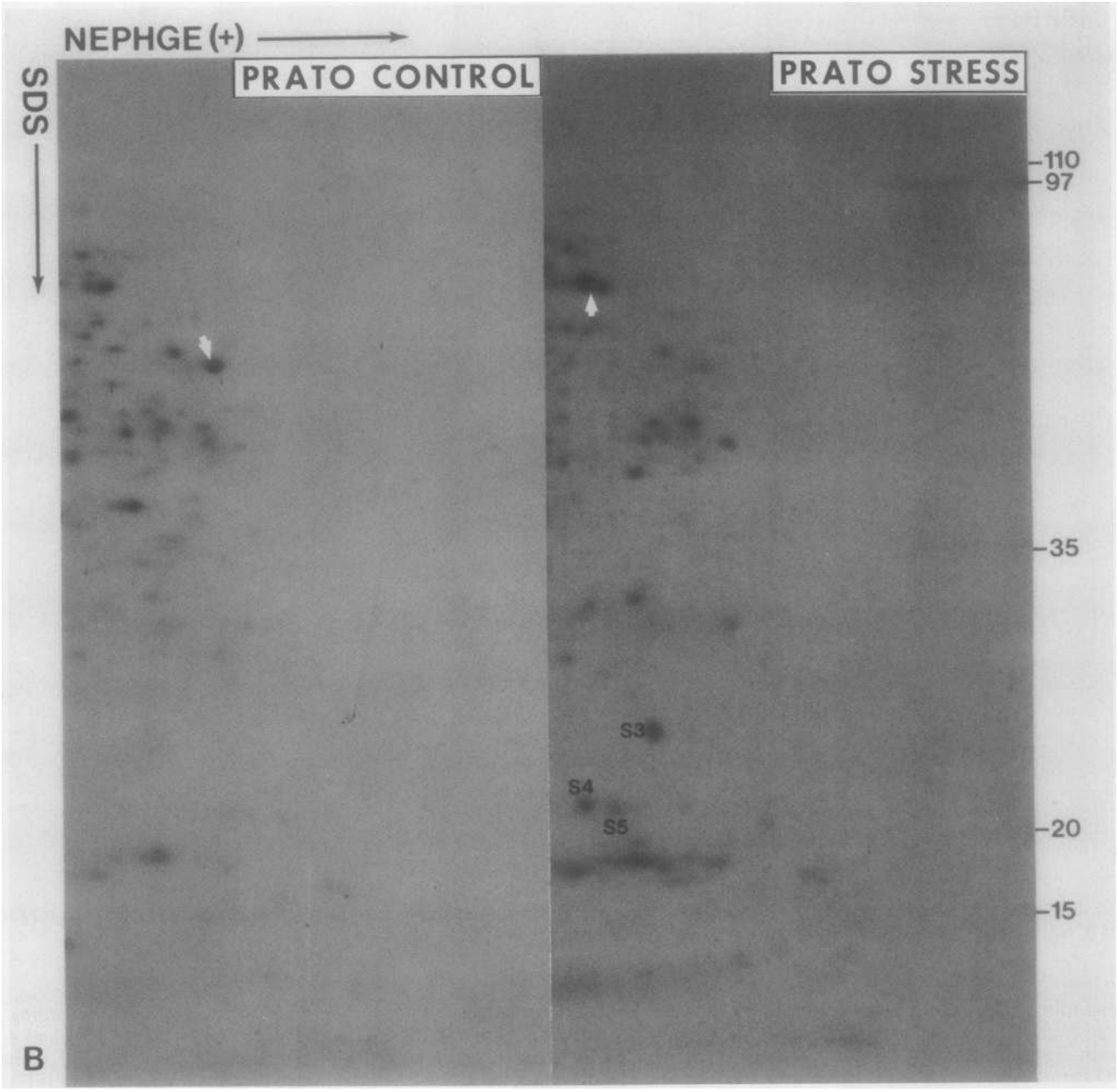

FIG. 2B.

appeared when the treatment was extended to 24 h. Incorporation was much lower at 3% NaCl than at 2% NaCl. For detailed analysis of individual proteins by 2-D gels, we restricted the treatment to 2% NaCl. Seedlings exposed to 2% NaCl for 20 h were able to recover as indicated by growth of new root hairs and resume normal levels of protein synthesis within 24 h of removal of NaCl (not shown).

The uptake of ^{35}S-methionine and its incorporation into proteins in root and shoot tissues are shown in Table I. Salt treatment inhibited the uptake of ^{35}S-methionine in both genotypes. The extent of reduction was higher in root tissue of CM72 than that of Prato but, in shoots, it was similar in both genotypes. The data also suggest inherent differences for methionine uptake in the two barley genotypes; CM72 was apparently more efficient than Prato. A comparison of root protein synthesis, corrected for differences in label uptake between the genotypes, suggests that it was slightly more sensitive to salt in Prato than in CM72. Interestingly, if the incorporation values were normalized for uptake differences induced by salt treatment, the data suggest that protein synthesis was actually stimulated by salt treatment in CM72. However, further experiments are necessary to understand this stimulation. In shoots, protein synthesis was more sensitive to salt than in roots. The data, corrected for uptake variations due to genotype as well as salt treatment, indicate that salt reduced shoot protein synthesis in Prato slightly more than in CM72.

When the salinity induced changes in 2-D patterns of steady state proteins of roots and shoots in the two cultivars after staining the gels with Coomassie blue or silver were examined no detectable differences were found (data not shown). Also, the protein composition of the two cultivars was almost identical in root and shoot tissues (13). However, an analysis of the newly synthesized proteins indicated many significant differences.

Seedlings exposed to NaCl and controls were labeled with ^{35}S-

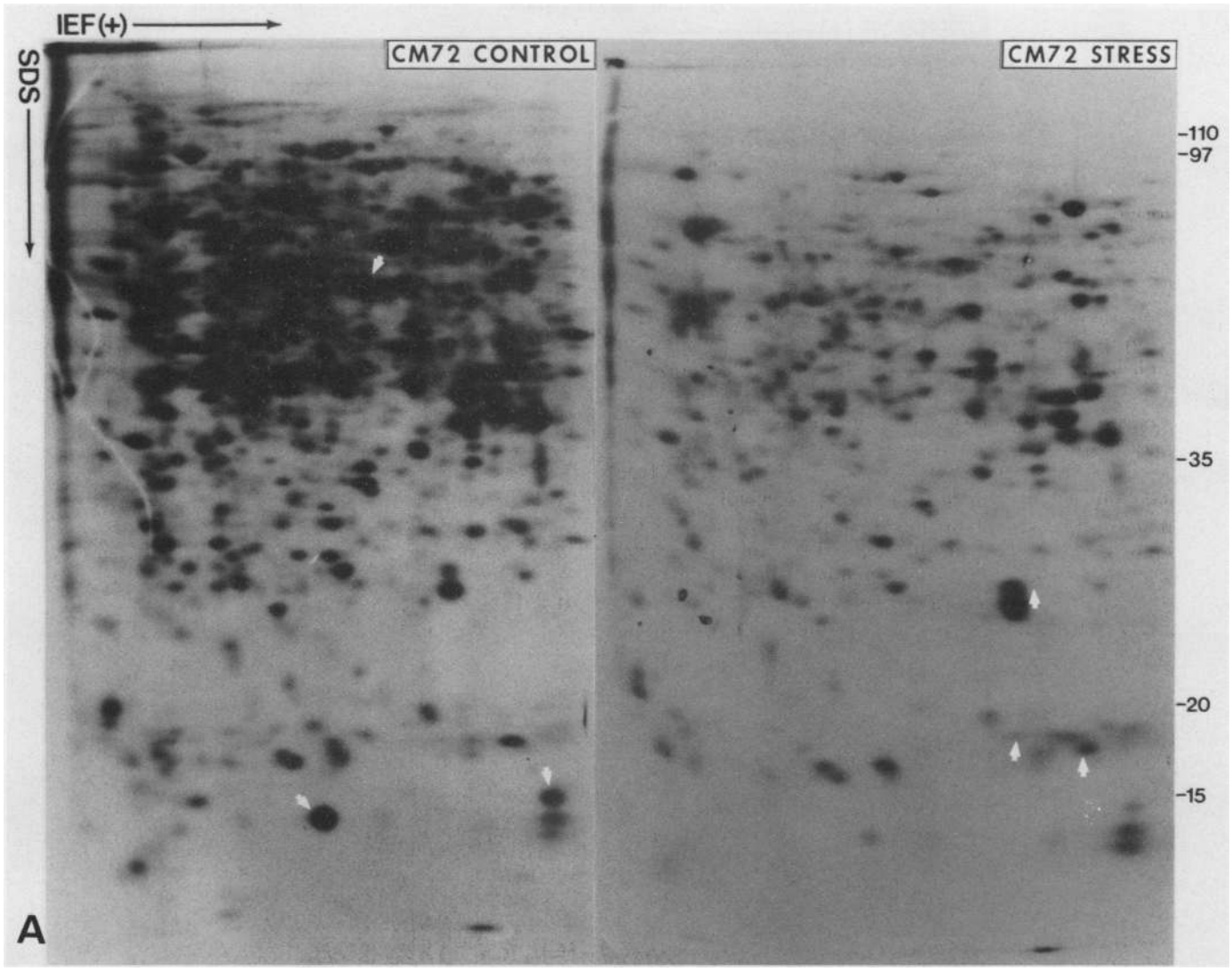

FIG. 3. Pattern of newly synthesized shoot proteins in barley seedlings (CM72). Details are as described in Figures 1 and 2. Panel A is IEF/2-D; panel B, NEPHGE/2-D. S3 and S4 are the only new shoot proteins induced by stress in this genotype.

methionine and the newly made proteins resolved by IEF/2-D and NEPHGE/2-D. The pattern of root proteins in CM72 is presented in Figure 1, panels A and B. NaCl treatment resulted in the following specific changes in root proteins: (a) The synthesis of several proteins (about 15 as shown in Fig. 1, control panels) declined. (b) The specific synthesis of about 10 proteins was enhanced (Fig. 1, stress panels). (c) Six new proteins (R1–R6) were induced apparently (Fig. 1, stress panels); occasionally trace of protein R4 (26.5 kD) was also seen in the controls. The apparent M_r and pI data for each of these proteins are summarized in Table II.

Salinity induced root protein changes were identical in CM72 and Prato except that one protein (marked × in Fig. 1, control panel) was consistently undetectable in the latter cultivar (also absent in controls). Similar results were obtained regardless of whether the roots were exposed for 6 or 18 h to NaCl.

The patterns of newly synthesized shoot proteins in Prato and CM72 are shown in Figures 2 and 3, respectively. It is evident from the protein pattern that NaCl reduced the synthesis of all of the proteins to the same extent except, for example, about 4 as indicated by arrows in the control panels of Figures 2 and 3, which seemed to be reduced even greater than the remainder; the preferential synthesis of about 5 proteins (Figs. 2 and 3, stress panels, arrows) was also enhanced as in roots.

Five new shoot proteins whose distribution showed distinct differences between the two cultivars were induced by salinity. Proteins S1, S2, and S5 were unique to Prato (Fig. 2). Proteins S3 and S4 were synthesized in both CM72 and Prato (Figs. 2 and 3). The properties of these proteins are summarized in Table II. As with roots, the synthesis of these proteins was apparent after 6 h of exposure to NaCl as well as after 18 h of treatment.

The protein pattern in seedlings recovering from the stress treatment was also investigated (data not shown). Seedlings treated for 18 h with 2% NaCl were allowed to recover for 21 h in the absence of NaCl. In these seedlings, the salinity-induced quantitative variation in synthesis of several proteins disappeared but a low level synthesis of R1, R2, R3, R4, and R6 was still detectable. Shoot proteins were not analyzed.

DISCUSSION

Investigations on gene expression induced by salinity stress in higher plants are limited compared to studies on heat shock and anaerobic stress. Protein expression altered by salinity treatment

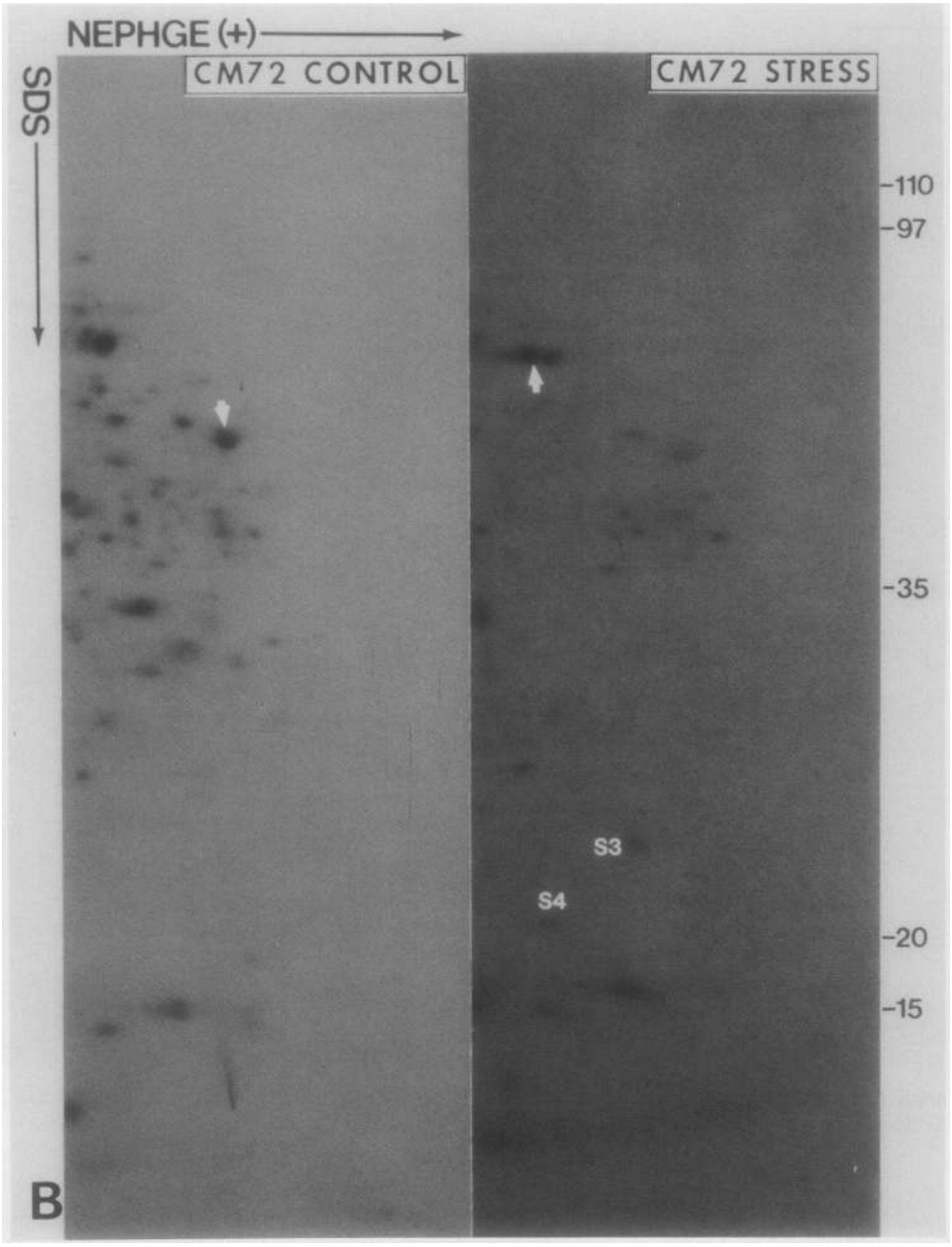

FIG. 3B.

has been studied in tissue culture systems of tobacco (5, 19) and maize (12). Work in a tobacco cell culture system using primarily single dimensional SDS-PAGE and Coomassie blue staining showed salinity induced changes in the concentration of many polypeptides (5, 19). Two proteins of 26 and 43 kD were apparently unique to salt-adapted cells. However, when the 26 kD protein was studied in detail by incorporating ^{35}S, its synthesis was detected in both unadapted and adapted cultures (19). In the maize culture, seven newly synthesized proteins were regulated by salinity stress; three of these (74, 28.5, and 26.2 kD) were apparently induced *de novo* (12).

In this study, I have employed different 2-D gel electrophoretic techniques to resolve salinity induced changes in all of the extractable barley proteins. Discernible differences in the two cultivars were found only in the newly synthesized protein populations. Two major effects of salinity stress are noteworthy. First, there was a quantitative regulation of the synthesis of individual proteins. The synthesis of a wide spectrum of proteins of different M_r and pI was either enhanced or curtailed by salinity. Second, a coordinate induction of a select group of new proteins was found. Overall, salinity induced six new root proteins and five new shoot proteins. Two of the general features of these proteins were that they all fell within a narrow M_r group of 20,000 to 30,000 and a pI range of 6.1 to 7.6. Because of the apparent low M_r of salinity induced proteins and the predominant induction of low M_r heat shock proteins in plants (7), the relationship between the proteins induced by these two stress signals was compared. The data revealed that stress proteins induced by salinity and heat shock were distinctly different in barley and differed with respect to pI and M_r (13).

Salinity tolerance in plants is considered as a polygenic trait and the expression of tolerance may be dependent upon tissue and/or developmental stage (3, 18). The present study shows that the root and shoot proteins altered by salinity in the seedling

stage are distinct in their electrophoretic properties and therefore, are apparently unique to each tissue. This is in contrast to the findings on the tissue-specific distribution of heat shock proteins in maize seedlings in which a comparison of different parts of the plant showed that the same set of identical proteins was induced in each tissue (2).

To understand the molecular basis for mechanisms of salt tolerance in plants, it is important to distinguish constitutive and induced differences among genotypes, especially in those which show differential responses. The present and other data (13) indicate that protein expression in various tissues was identical in the two barley genotypes when no stress was imposed. However, as demonstrated in this study, salinity stress elicited genotype-specific protein changes in shoots but not in roots. Two new proteins were induced in both the salt-tolerant and -sensitive cultivars but an additional three unique proteins were made only in the latter. In a study of several maize genotypes during heat shock, the same six heat shock proteins were induced in the various tissues of all varieties (2). However, the maize varieties were apparently not selected for differences in thermotolerance.

Both transcriptional and posttranscriptional mechanisms could account for the expression of proteins during salinity stress in the salt-tolerant and salt-sensitive barley genotypes. These mechanisms are considered in a forthcoming paper (14).

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