Plant Physiol. (1989) 89, 577-585
0032-0889/89/89/0577/09/$01.00/0

Received for publication March 3, 1988
and in revised form August 26, 1988

Synthesis of Freezing Tolerance Proteins in Leaves, Crown, and Roots during Cold Acclimation of Wheat[1]

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ABSTRACT

Protein synthesis was studied in leaves, crown, and roots during cold hardening of freezing tolerant winter wheat (*Triticum aestivum* L. cv Fredrick and cv Norstar) and freezing sensitive spring wheat (*T. aestivum* L. cv Glenlea). The steady state and newly synthesized proteins, labeled with [^{35}S]methionine, were resolved by one- and two-dimensional polyacrylamide gels. The results showed that cold hardening induced important changes in the soluble protein patterns depending upon the tissue and cultivar freezing tolerance. At least eight new proteins were induced in hardened tissues. A 200 kilodalton (kD) (isoelectric point [pI] 6.85) protein was induced concomitantly in the leaves, crown, and roots. Two proteins were specifically induced in the leaves (both 36 kD, pI 5.55 and 5.70); three in the crown with M_r 150 (pI 5.30), 45 (pI 5.75), and 44 kD (pI > 6.80); and two others in the roots with M_r 64 (pI 6.20) and 52 kD (pI 5.55). In addition, 19 other proteins were synthesized at a modified rate (increased or decreased) in the leaves, 18 in the crown and 23 in the roots. Among the proteins induced or increased in hardened tissues, some were expressed at a higher level in the freezing tolerant cultivars than in the sensitive one, indicating a correlation between the synthesis and accumulation of these proteins and the degree of freezing tolerance. These proteins, suggested to be freezing tolerance proteins, may have an important role in the cellular adaptation to freezing.

The underlying physiological and molecular mechanisms of freezing tolerance of plants are poorly understood. It has been suggested that the development of cold resistance may depend on altered gene expression and the synthesis of new proteins. Recently, we reported on changes in the soluble protein patterns of cold hardened etiolated wheat seedlings (15). A high mol wt protein in the range of 200 kD appeared in hardened plants and its accumulation was correlated with freezing tolerance. Guy and Haskell (5) have also found three high mol wt induced proteins (160, 117, and 85 kD) in spinach during cold acclimation. On the other hand, Mohapatra *et al.* (10) reported the appearance of several small polypeptides ranging from 11 to 38 kD in cold acclimated alfalfa seedlings. Using cell suspension cultures, Johnson-Flanagan and Singh (7) working with winter rape and Robertson *et al.* (13) with bromegrass, found low mol wt peptides ranging from 20 to 48 kD during the induction of freezing tolerance. These discrepancies could be due to species differences or to the different experimental approaches. However, it is difficult to determine whether these changes are associated with growth at low temperature or with the development of freezing tolerance. Thus, more detailed studies are needed to understand the exact relationship between protein synthesis and freezing tolerance. In this report, we studied *in vivo* protein synthesis in leaves, crown, and roots of three wheat genotypes which differ in their low temperature response and their capacity to develop freezing tolerance. These comparisons provide additional information on the molecular and genetic basis of the cold acclimation process in cereal plants.

MATERIALS AND METHODS

Plant Material

Spring wheat (*Triticum aestivum* L. cv Glenlea) and winter wheat (*T. aestivum* L. cv Fredrick and Norstar) seeds were germinated in moist sterilized vermiculite for 5 d in the dark and 2 d under artificial light. Cool-white fluorescent and incandescent lighting was combined to provide an irradiance of 450 μmol m^{-2} s^{-1}. The temperature was maintained at 24 ± 1°C (15 h photoperiod) during the day and 20 ± 1°C during the night. The RH was 70 ± 5%. Seedlings were watered daily with Hoagland nutrient solution (1). After germination, control plants were maintained under the same conditions of light and temperature for 7 d. Hardening was performed by subjecting germinated seedlings to a temperature of 6 ± 1°C during the day (10 h photoperiod) and 2 ± 1°C night for 40 d. Based on dry weight, control seedlings of 9 and 14 d correspond in physiological age to seedlings hardened respectively for 10 and 40 d (12).

Evaluation of Freezing Tolerance

Freezing tolerance of whole seedlings was determined as reported previously (12) and expressed as the temperature required to kill 50% of the seedlings (LT_{50}[2] °C), as indicated after a 2-week regrowth period.

Freezing tolerance of leaves, crowns, and roots was estimated by the electrolyte leakage method as described by Chen *et al.* (3) with slight modifications. Unhardened and hardened plants were carefully removed from the growing medium and thoroughly washed with distilled water. Leaves and roots were cut into 2 cm sections and crowns were cut 2 mm below and 1.2 cm above the apex. Samples of 20 leaf or root segments

[1] Supported by Natural Sciences and Engineering Research Council of Canada grant A 7199 to F. S.

[2] Abbreviations: LT_{50}, 50% killing temperature; FTP, freezing tolerance protein; IEF, isoelectric focusing.

and 10 crowns were placed in a freezer and cooled at 1.5°C h^{-1}. At various temperatures, samples were taken out of the freezer and slowly thawed before adding 10 mL of ice-cold distilled water. Samples were then vacuum infiltrated, shaken, and warmed to room temperature, and the conductivity of the leachate was measured with a conductivity meter. To obtain total leachate, tissues were autoclaved for 20 min in sealed tubes and the conductivity of the leachate was determined again. Freezing tolerance was expressed as the temperature which caused 50% ion leakage.

In Vivo Labeling of Proteins

Ten (10) seedlings, carefully removed from vermiculite to avoid root damage, were washed extensively with running water followed by sterile water. To minimize the transplantation stress, the seedlings were kept overnight in sterile Hoagland nutrient solution supplemented with 50 μg/mL chloramphenicol as a precaution against any bacterial growth. Seedlings were labeled in 10 mL of sterile water containing 250 μCi of [^{35}S]methionine 1095 Ci/mmol (ICN). Labeling was carried out for 6 h at 24°C for control plants and 24 h at 6°C for cold hardened plants. After labeling, seedlings were washed with sterile water, and the leaves, crowns, and roots were sampled and quickly frozen at −70°C.

Protein Extraction

Frozen tissues (0.5 g) were ground with dry ice in a mortar and homogenized at 4°C in 5 mL of 100 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% (w/v) insoluble PVP, 50 mM DTT, and 1 mM PMSF. The homogenate was centrifuged at 165,000*g* for 90 min in a Beckman 50.1 rotor. Proteins of the soluble fraction were precipitated with 5 volumes of cold acetone at −20°C, and the pellet resuspended in either Laemmli (8) sample buffer (60 mM Tris-HCl [pH 6.8], 10% [w/v] glycerol, 2% [w/v] SDS, 5% 2-mercaptoethanol) or in O'Farrell (11) lysis buffer (9 mM urea, 2% ampholines [pH 3-10], 2% Nonidet P-40, 5% 2-mercaptoethanol) for two-dimensional electrophoresis. Aliquots of labeled proteins were mixed with scintillation liquid and then counted to determine the amount of [^{35}S]methionine incorporated into proteins. Protein concentrations were measured by the Lowry (9) method with BSA as standard.

Discontinuous SDS-PAGE (8)

Equal amounts of proteins (50 μg) were loaded on 8% polyacrylamide slab gels (4% stacking gel) and subjected to electrophoresis under constant current of 20 mA per gel. Gels were fixed and stained with 0.1% (w/v) Coomassie blue prepared in 50% (v/v) methanol and 10% (v/v) acetic acid. They were destained in 30% methanol and 7% acetic acid.

Two-Dimensional Gel Electrophoresis

In vivo labeled proteins were separated as described by O'Farrell (11) with the following modifications. The IEF gel electrophoresis was carried out in 140 × 1.5 mm tube gels containing 1.33% (v/v) ampholines (pH 5-7) (Bio-Rad) with 0.67% (v/v) ampholines (pH 3-10). Equal amounts of radioactivity (4 × 10^5 cpm) were loaded on each gel. After focusing for 14 h at 400 V followed by 1 h at 800 V, the gels were removed, preequilibrated for 30 min in Laemmli buffer, and subjected to the second dimension in the discontinuous SDS-PAGE system described before. Proteins of known mol wt (Bio-Rad) were run in parallel with the sample. Duplicate IEF gels were used to determine the pH scale.

Gel Staining and Autoradiography

Following electrophoresis, the gels were fixed and lightly stained with Coomassie blue in order to visualize the mol wt standards. The fixed and stained gels were dried and exposed to Kodak XAR-5 film at −70°C for 12 d.

RESULTS

Freezing Tolerance

Wheat seedlings subjected to appropriate hardening conditions of light and temperature developed an increased freezing tolerance, depending on the capacity of each genotype. When fully hardened (after 40 d at 6/2°C) the cold tolerant cultivars Norstar and Fredrick reached an LT_{50} of −19 and −16°C, respectively, while the cold sensitive cultivar Glenlea only resisted to −8°C (Table I). Unhardened control plants of similar growth stage (14 d at 24°C/20°C) were killed at temperatures slightly below zero with LT_{50}s ranging from −3 to −6°C for the three cultivars.

The freezing tolerance of aerial tissues increased significantly upon cold hardening and showed similar or higher values than those of the whole seedlings (Table I). Hardened leaf and crown tissues of the two winter cultivars (Fredrick and Norstar) showed LT_{50} values ranging from −14 to −21°C as compared to −6 to −7°C for control tissues. On the other hand, the freezing tolerance of the roots showed little change during the hardening treatment. No pronounced differences were observed among the three cultivars.

Changes in Soluble Protein Patterns

Separation of wheat shoot soluble proteins on SDS polyacrylamide gel is shown in Figure 1. Major differences be-

Table I. *Freezing Tolerance of Leaves, Crowns, Roots and Whole Seedlings of Wheat*

Tissues and whole seedlings were frozen to various temperatures, and tolerance determined by either the conductivity method for leaves, crowns, and roots or by the regrowth method for whole seedlings. Freezing tolerance is expressed as 50% killing temperature (LT_{50}, °C), as determined either by the ion leakage or the lack of regrowth. Values are means of three separate experiments.

Tissue	Unhardened			Hardened		
	G[a]	F	N	G	F	N
	LT_{50}, °C					
Leaves	−5	−6	−7	−8	−14	−17
Crown	−6	−7	−7	−9	−17	−21
Roots	−4	−5	−5	−4	−5	−6
Seedlings	−3	−4	−6	−8	−16	−19

[a] G, Glenlea; F, Fredrick; N, Norstar.

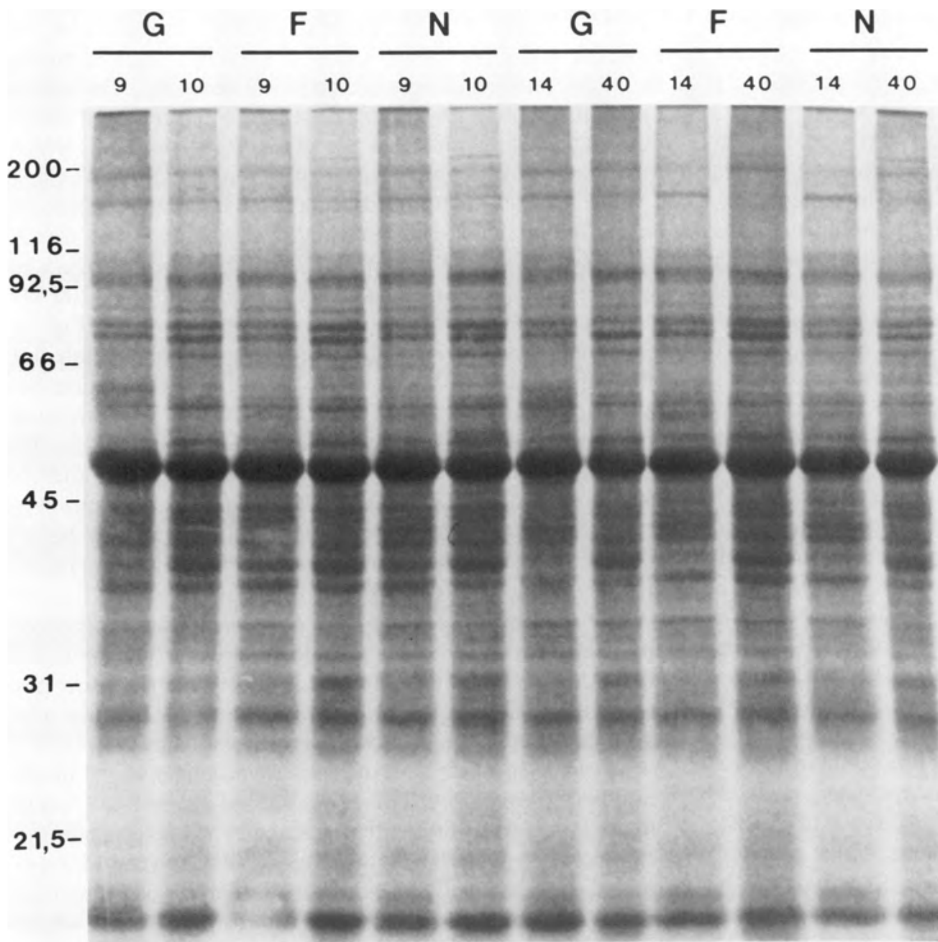

Figure 1. SDS-PAGE of soluble protein fractions from three wheat cultivars (Glenlea, G; Fredrick, F; and Norstar, N) hardened for 10 and 40 d (lanes 10 and 40). Lanes 9 and 14 correspond to unhardened seedlings of similar growth stages. Proteins were solubilized in Laemmli buffer, electrophoresed on an 8% polyacrylamide gel, and stained with Coomassie blue. $M_r \times 10^{-3}$ of standard proteins are shown on the left. Full arrows indicate bands which were induced or increased in intensity in hardened plants. Open arrows indicate bands which were repressed or decreased in intensity in hardened plants.

tween hardened and unhardened seedlings were apparent after 10 d of hardening and these changes were more pronounced following a longer hardening period. The analysis of the protein patterns revealed the accumulation of a high mol wt protein of 200 kD which appeared to be induced by the low temperature hardening. This band was particularly evident in the two cold tolerant cultivars Norstar and Fredrick, but less apparent in the cold sensitive cultivar Glenlea. In addition, eight protein bands (180, 77, 74, 68, 52, 43, 38, and 31 kD) increased in intensity in tissues subjected to hardening conditions, while the content of three other bands (157, 42, and 34 kD) decreased. The 157 kD protein almost disappeared at the end of the hardening period (40 d), particularly in the two resistant cultivars (Fig. 1, F40 and N40).

In Vivo Protein Synthesis

The uptake of [^{35}S]methionine and its incorporation into leaf, crown, and root proteins are shown in Table II. The distribution of the [^{35}S]methionine in the different tissues showed no differences between control and hardened plants of the resistant cultivars Fredrick and Norstar. On the other hand, the sensitive cultivar seemed to accumulate and incorporate more [^{35}S]methionine at the leaf level upon exposure to low temperature.

The amount of methionine incorporated by hardened plants during 24 h of labeling at 6°C was similar or higher than that incorporated by control plants during 6 h at 24°C.

Table II. *In Vivo Labeling of Hardened and Unhardened Wheat Seedlings*

Seedlings were labeled with [^{35}S]methionine as described in "Materials and Methods." Distribution of the radioactivity within the different tissues is given as percentage of the total amount accumulated and/or incorporated into proteins per seedling. The results are averages of duplicate determinations.

Tissue	Accumulation			Incorporation		
	G[a]	F	N	G	F	N
	% of total amount					
Unhardened						
Leaves	11.5	25.2	16.0	12.1	26.3	17.2
Crown	25.9	30.5	18.9	27.4	35.1	21.5
Roots	62.6	44.3	65.1	60.4	38.5	61.3
Total	(33.3)[b]	(29.5)	(31.6)	(19.6)	(18.5)	(18.4)
Hardened						
Leaves	22.8	23.1	15.0	25.2	23.5	17.4
Crown	22.4	35.0	18.6	25.4	40.6	22.6
Roots	54.7	41.9	66.4	49.4	35.9	60.0
Total	(42.6)	(33.4)	(34.6)	(27.6)	(22.1)	(23.4)

[a] G, Glenlea; F, Fredrick; N, Norstar. [b] Numbers in parentheses represent the total amount of [^{35}S]methionine (dpm $\times 10^{-6}$) accumulated or incorporated into proteins per seedling.

This result showed that the protein synthesis activity remained relatively high at hardening temperature considering the lower growth rate of the seedlings (five d at 6/2°C correspond to 1 d at 24/20°C [2]).

Labeled soluble proteins from cold hardened and unhardened tissues (leaf, crown, and root) of three wheat cultivars were analyzed using two-dimensional gel electrophoresis. Figure 2 and Table III illustrate the changes in protein synthesis in the leaves of the three cultivars after 40 d of hardening compared to unhardened plants of similar physiological age. From the comparison, we found that cold hardening induced the synthesis of at least three new proteins, a high mol wt polypeptide of 200 kD (spot A, pI 6.85) and two low mol wt peptides of 36 kD (spots H and I, pI 5.55 and 5.70). In addition, 7 proteins were synthesized in greater amounts and 12 others decreased in intensity in cold hardened plants.

Numerous changes also occurred in the protein patterns of the crown tissues subjected to cold hardening conditions (Fig. 3). Four proteins (spots A [200 kD, pI 6.85]; B [150 kD, pI 5.30]; J [45 kD, pI 5.75]; and K [44 kD, pI > 6.80]) were induced by the hardening conditions. Two proteins were repressed (spots 2 and 8; 60 and 41 kD, respectively) and 16 others were synthesized at a modified rate (intensity of the spots increased or decreased as compared to the unhardened ones).

The protein patterns of root tissues are illustrated in Figure 4. The results showed that the roots were appreciably affected by the low temperature of hardening. The synthesis of at least 26 proteins was modified in hardened plants. Three proteins were induced (spots A [200 kD, pI 6.85]; G [64 kD, pI 6.20]; and J [52 kD, pI 5.55]), 13 increased in intensity, 4 decreased and 6 were repressed by the hardening conditions (spots 1-3, 6, 7, and 9; 60, 53, 46, 39, 38, and 33 kD).

A number of proteins in the leaves (Fig. 2; spots A, G, H, I), in the crown (Fig. 3; spot A) and in the roots (Fig. 4; spots A, G, J, K, M) were synthesized at a higher rate in cold tolerant cultivars than in the sensitive one. This was particularly evident with the 200 kD protein (spot A) which was one of the major labeled spots in hardened Fredrick and Norstar leaves and crowns, but was barely visible in the cold sensitive Glenlea. In contrast, some proteins (see, for example, spots X1 and X2 in Fig. 2) increased in intensity in hardened leaves of the cold sensitive Glenlea cultivar while remaining stable or slightly decreasing in intensity in cold tolerant cultivars Fredrick and Norstar. This differential labeling pattern in the cold tolerant cultivars may be related to their ability to develop a greater resistance to freezing.

DISCUSSION

Cold hardening in higher plants is a complex process involving many genes, none of which has been identified. The nature of the adaptation process and the intensity of the response varies among plant species and among the different cultivars of wheat. The comparison of the gene expression in different genotypes, varying in their degree of freezing tolerance, appears to be a valid way to identify the modifications specifically related to the development of freezing resistance from those related to the low temperature stress. Using this approach, we were able recently (15) to associate the accumulation of a high mol wt polypeptide of 200 kD with the cold hardening of etiolated wheat seedlings. In this report, we present a comprehensive study of protein synthesis during cold acclimation of light-grown wheat seedlings. The results demonstrate that low temperature hardening triggered differential protein synthesis in the three genotypes used, which may indicate differential gene expression.

The analysis of the steady state protein patterns of shoots showed that cold hardening induced modifications in the expression of at least 12 peptides. The most noticeable changes were the induction of a high mol wt polypeptide of 200 kD and the repression of a 157 kD in hardened seedlings (Fig. 1). These changes were more pronounced in the two cold tolerant cultivars Fredrick and Norstar, indicating a correlation between the synthesis of these peptides and the degree of freezing tolerance of the different genotypes. The induction of the 200 kD protein in the early stages of cold hardening and its accumulation throughout the whole hardening process suggests that this low temperature induced protein may be required for the acquisition, the development and/or the maintenance of the increased freezing resistance in wheat.

The soluble protein patterns also revealed that eight other peptides, ranging from 31 to 180 kD, increased in all cultivars upon low temperature exposure. These changes were not correlated with the level of cultivar freezing tolerance and appeared to result from growth at low temperature.

The analysis of the *in vivo* protein synthesis revealed that a number of proteins were newly expressed or repressed, while many others were differently regulated by the cold hardening conditions (Table III). At least eight new polypeptides appeared in hardened tissues. The appearance of these new peptides in the protein synthesis patterns of hardened wheat tissues suggested that cold acclimation induced modifications at the gene expression level. It is not clear at this point whether these changes took place at the transcriptional or translational level. Modifications of gene expression during cold acclimation had also been observed recently in spinach (5, 6), alfalfa (10), winter rape (7), and bromegrass (13).

Among the polypeptides induced by the hardening conditions, some were synthesized at a higher rate in the two cold tolerant cultivars than in the cold sensitive one, indicating a correlation between the degree of expression of these genes and the degree of tolerance reached by the different genotypes. These proteins (spot A [200 kD] in the three tissues, spots H [36 kD] and I [36 kD] in the leaves, and spots G [64 kD] and J [52 kD] in the roots) were therefore considered as part of a potential family of FTPs, *e.g.* proteins strongly associated with the development and the maintenance of the increased freezing resistance. On the other hand, some proteins already present in control tissues increased in intensity in hardened tissues of the three cultivars (Table III), but could not be correlated with the degree of freezing tolerance. These proteins were more likely to belong to a protein family involved in the adjustment of the plant metabolism to growth at low temperature. These proteins would ensure cell survival at low temperatures rather than increasing their ability to tolerate freezing and, therefore, should be distinguished from the putative FTPs. The differential expression of the freezing tolerance genes in the different genotypes could be due to the number of active copies (gene amplification?) or to the presence of specific factor(s) which regulate differentially the transcrip-

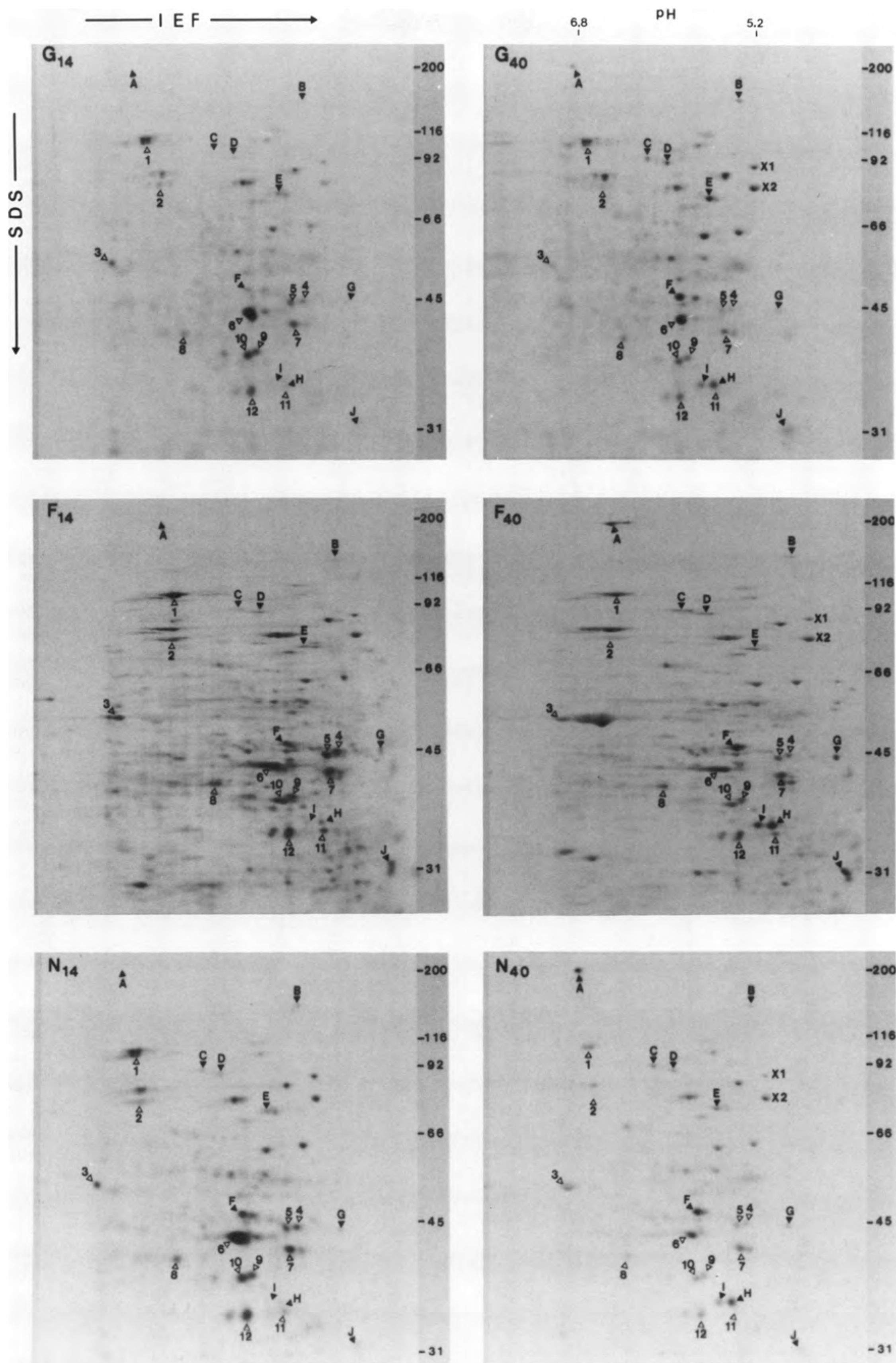

Figure 2. Patterns of newly synthesized leaf proteins of hardened and unhardened wheat seedlings (cv Glenlea, G; cv Fredrick, F; and cv Norstar, N). Labeled soluble proteins were prepared and resolved by IEF/SDS-PAGE as described in "Materials and Methods." G_{40}, F_{40}, and N_{40}, hardened for 40 d. G_{14}, F_{14}, and N_{14}, unhardened control. Full arrowheads with letters indicate spots which were induced or increased in intensity in hardened tissues. Open arrowheads with numbers indicate spots which were repressed or decreased in intensity in hardened tissues. (Spots X1 and X2 show peptides differently regulated in sensitive and tolerant cultivars. (See text.) Gels were loaded with 4×10^5 cpm of cold acetone-insoluble radioactivity. $M_r \times 10^{-3}$ of standard proteins are indicated. pH scale, determined on duplicate gels, was linear between pH 5.2 and pH 6.8.

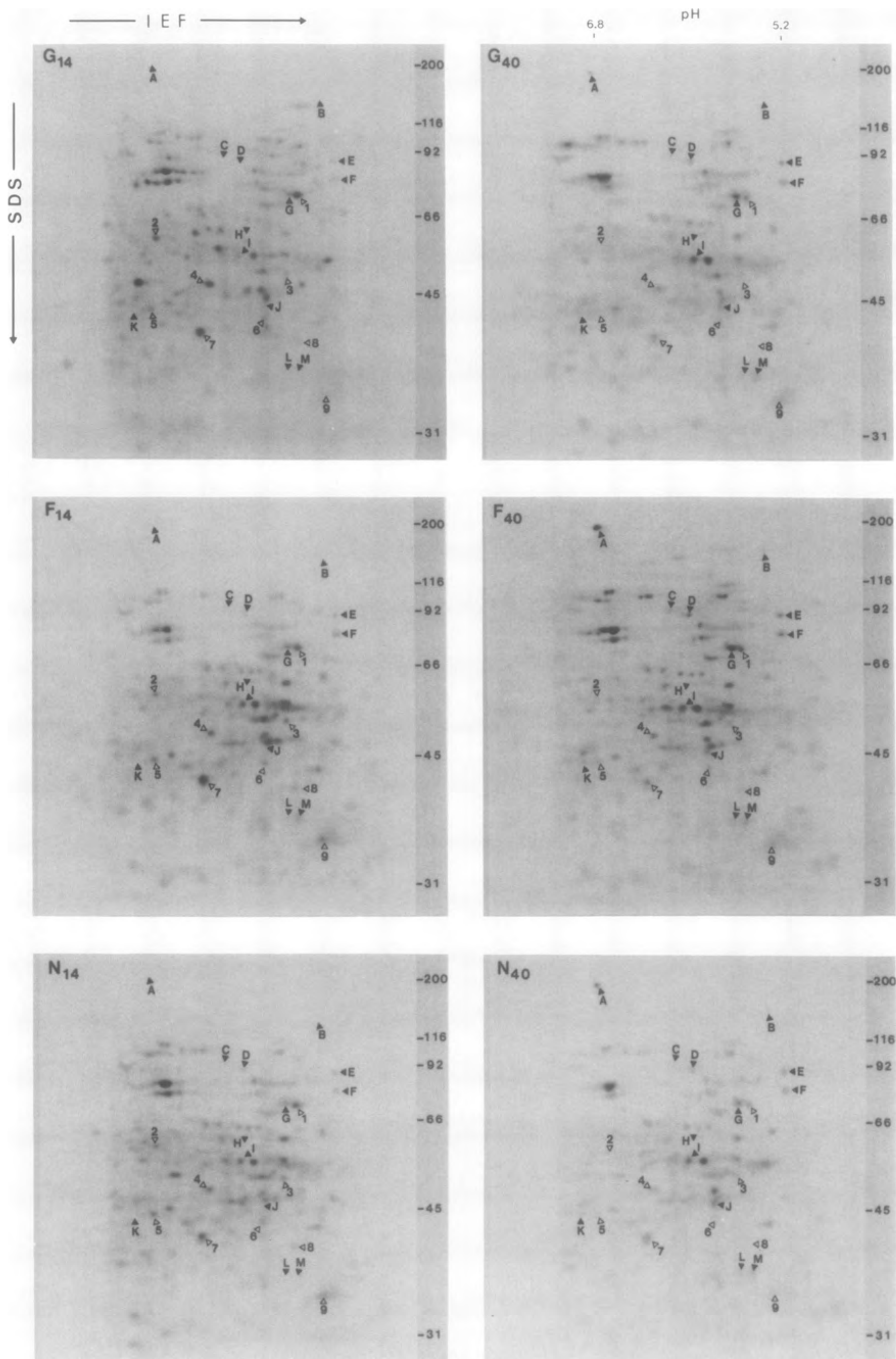

Figure 3. Patterns of newly synthesized crown proteins of hardened and unhardened wheat seedlings (cv Glenlea, G; cv Fredrick, F; and cv Norstar, N). Details as in legend to Figure 2.

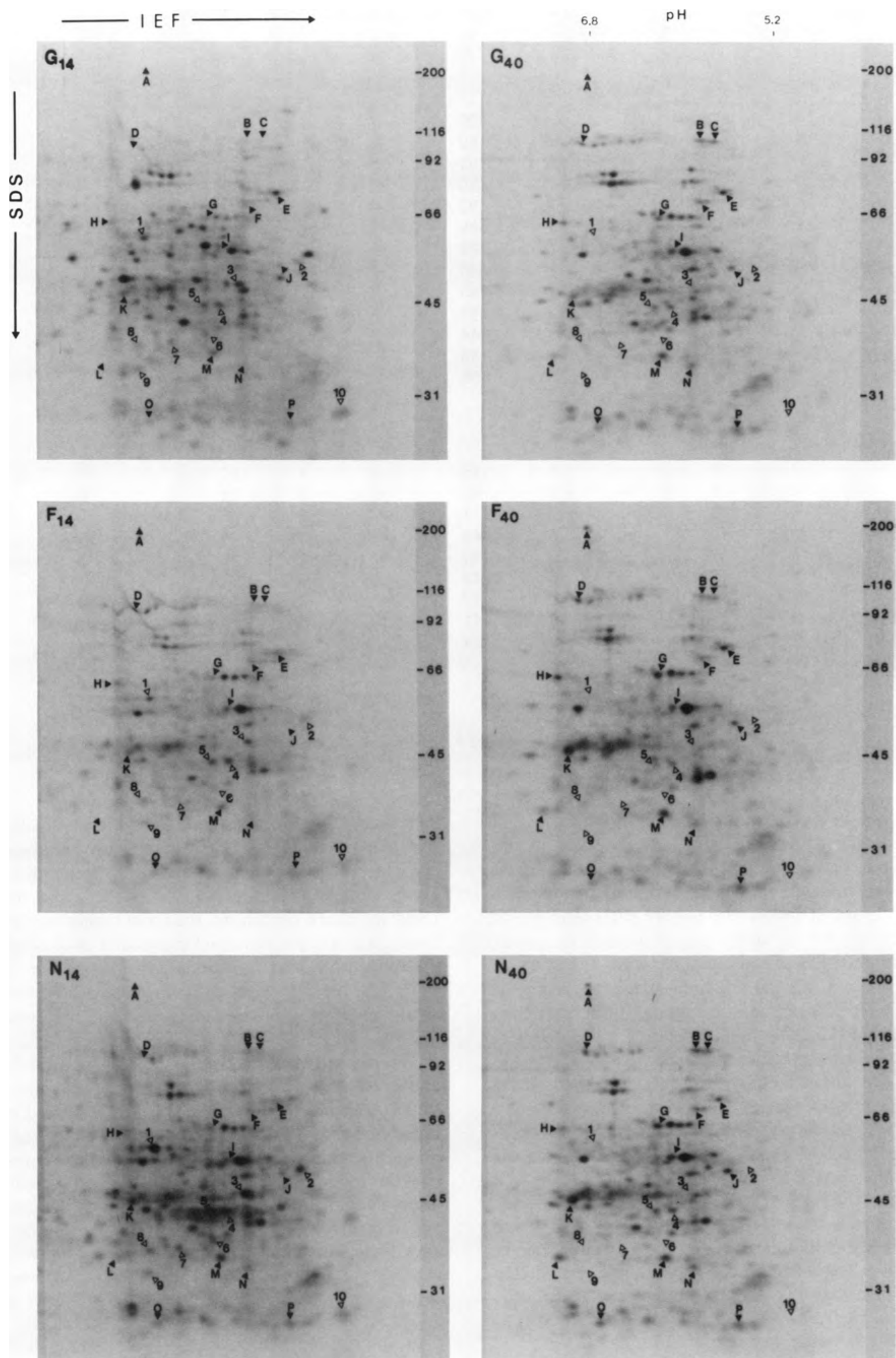

Figure 4. Patterns of newly synthesized root proteins of hardened and unhardened wheat seedlings (cv Glenlea, G; Fredrick, F; and cv Norstar, N). Details as in legend to Figure 2.

Table III. *Analysis of In Vivo Labeled Proteins*

Leaves			Crown			Roots		
Spot[a]	$M_r \times 10^{-3}$	Regulation[b]	Spot	$M_r \times 10^{-3}$	Regulation	Spot	$M_r \times 10^{-3}$	Regulation
A	200	i	A	200	i	A	200	i
B	150	+	B	150	i	B	107	+
C	92	+	C	92	+	C	106	+
D	90	+	D	90	+	D	105	+
E	75	+	E	90	+	E	75	+
F	48	+	F	80	+	F	71	+
G	45	+	G	75	+	G	64	i
H	36	i	H	60	+	H	63	+
I	36	i	I	55	+	I	55	+
J	31	+	J	45	i	J	52	i
			K	44	i	K	45	+
			L	36	+	L	36	+
			M	36	+	M	36	+
						N	34	+
						O	28	+
						P	27	+
1	105	−	1	75	−	1	60	r
2	79	−	2	60	r	2	53	r
3	53	−	3	51	−	3	46	r
4	45	−	4	49	−	4	44	−
5	44	−	5	44	−	5	43	−
6	43	−	6	43	−	6	39	r
7	42	−	7	42	−	7	38	r
8	40	−	8	41	r	8	37	−
9	38	−	9	34	−	9	33	r
10	38	−				10	29	−
11	35	−						
12	34	−						

[a] Spots designation corresponds to that in Figures 2 to 4. [b] i, Induced; r, repressed; +, increased; −, decreased.

tion, translation, or protein turnover rate during hardening. The increase in DNA-dependent RNA polymerases activity observed in our earlier study (14), may be a result of the activation by such regulatory factors during hardening. This regulation at the transcription level may be part of the genetic control responsible for the differential expression of specific freezing tolerance mRNAs and their products.

The induction of a 200 kD protein as well as the increased synthesis of a 75 kD peptide concomitantly in all tissues suggested that part of the genetic regulation associated with the increased freezing tolerance took place at the whole plant level. The regulation at the plant level could depend on the ability of some tolerance genes to respond to a translocatable regulatory factor similar to the 'hardiness promoting factor' described by Fuchigami *et al.* (4) and Weiser (16). On the other hand, the induction of specific proteins in the leaf, crown, and root tissues indicated that the regulation of some freezing tolerance genes was tissue specific. This finding was supported by the fact that the synthesis rate of some proteins, detected in more than one tissue, was differently regulated in the different tissues during cold hardening (see, for example, the intensity of the spots H and I in Fig. 2 as compared to that of the corresponding spots L and M in Fig. 3).

We feel the most significant change detected in the protein synthesis patterns during cold hardening was the induction of the 200 kD freezing-tolerance protein in the leaf, crown, and root tissues. Comparing the relative intensity of the 200 kD spot with that of the other major spots, we could see that this specific protein was synthesized in greater amount in the leaves and the crown (aerial part of the plant) than in the roots. In natural conditions, the aerial tissues were exposed to lower temperatures and develop a greater cold resistance than the roots. Thus, the preferential synthesis and accumulation at the shoot level suggested a close correlation between the synthesis of the 200 kD protein and the tissue capacity to develop a significant freezing resistance.

The induction and accumulation of this protein did not appear to be associated with the plant developmental stages since it was induced in hardened light-grown seedlings (composed of 3 leaves) as well as in hardened etiolated epicotyls (15). The intensity of the 200 kD spot on the autoradiograms and the amount of [^{35}S]methionine incorporated into this protein suggested also that it was newly synthesized and did not result from posttranslational modifications of a protein already present. This conclusion is strongly supported by the fact that polysomes isolated from cold hardened wheat tissues contain a new mRNA coding for a high mol wt peptide of 200 kD (M Perras, F Sarhan, unpublished results).

In order to elucidate the nature, subcellular location, and the roles of the putative freezing tolerance proteins identified in this report, we intend to purify and characterize these specific peptides. This information should facilitate the iden-

tification and isolation of the corresponding genes and allow us to study their regulation during the development of cold hardening.

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