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# Changes in the Electrophoretic Patterns of the Soluble Proteins of Winter Wheat and Rye following Cold Acclimation and Desiccation Stress[1]

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## ABSTRACT

**The degrees of freezing tolerance acquired by winter wheat (*Triticum aestivium* L.) and rye (*Secale cereale* L. cv Puma) were similar following a 4-week cold conditioning and a 24-hour desiccation stress. Soluble proteins were extracted from shoots of cold-conditioned or desiccation-stressed seedlings and electrophoresed on sodium dodecyl sulfate-polyacrylamide gels. Quantitative changes in the electrophoretic patterns of the soluble proteins of the different cultivars grown in different environments were detected, but the changes were not equivalent following cold conditioning and desiccation stress. The abundance of two polypeptide bands showed a significant increase correlated to the degree of freezing tolerance and, hence, the polypeptides in these bands may play a role in the development of freezing tolerance.**

The plasma membrane has been suggested as the primary site of frost and plasmolysis dehydration injury of plant cells (7, 16, 18, 19). The mechanism by which the membrane of hardy cells tolerates more freezing and dehydration is not fully known but could be related to the general observation that metabolites accumulate in plant cells during hardening (17), a condition called augmentation of protoplasm (16).

The amount of soluble proteins increases in cold and desiccation-hardened plants (2, 4, 15) and soluble proteins during cold hardening also undergo changes in electrophoretic mobility (1, 5, 6, 9–12, 14). Soluble protein fractions with apparent mol wt of 240,000 and 115,000 in wheat (14) and of 30,000 and 25,500 in rape (12) accumulate during acquisition of frost tolerance. In alfalfa, 10 of the 14 polypeptide bands analyzed by Faw and Jung (9) increased whereas no changes are reported in hydrophobic proteins (1, 14).

In this report, we examined electrophoretic pattern changes of soluble proteins in relation to increased freezing tolerance following cold conditioning and desiccation stress. Detection of such changes in desiccation-stressed seedlings would provide information about the physiological equivalence or nonequivalence of desiccation stress and cold conditioning.

## MATERIALS AND METHODS

**Plant Materials and Growing Conditions.** Seed of six cultivars of winter wheat (*Triticum aestivium* L. cv Cappelle-Desprez, Monopol, Kent, Fredrick, Rideau, and Kharkov) and one of rye (*Secale cereale* L. cv Puma) were surface sterilized with 2.6% NaOCl for 3 min and imbibed at 21 or at 2°C (cold conditioned) for 6 h. Seeds were germinated on moist filter paper in the dark for 2.0 to 2.5 d at 24°C or for 4 weeks at 2°C (cold-conditioned). Seedlings were desiccation stressed by placing them for 24 h at 21°C over $H_2SO_4$ solution of 40% RH (3) and were reimbibed by immersion in aerated tap water for 24 h at 9°C.

**Evaluation of Freezing Tolerance.** Water was removed from the surface of seedlings by blotting with filter paper. Seedlings were frozen in groups of 25 in Petri dishes. Cooling rates were 1°C/h from 0 to −21°C and 3°C/h from −21 to −30°C. Some of the seedlings were further cooled to −40°C for 1 h and put on Dry Ice for 1 h additional. Samples were seeded with ice crystals at −3°C to avoid supercooling. Frozen samples were thawed at 2°C for 1 h. Survival of excised epicotyls was assessed by the method we described in a previous paper (3).

**Soluble Protein Extraction and Determination.** For each preparation of soluble proteins, 20 shoots were cut from fresh seedlings and weighed. Proteins were extracted by homogenization of the tissues in 0.1 M Tris-HCl (pH 7.0) at 0°C with a Polytron homogenizer and determined according to Siminovitch *et al.* (16).

**Electrophoresis and Calculation of Relative Abundance of Polypeptides.** TCA-precipitated soluble proteins were solubilized in the final sample buffer of Laemmli (13) and electrophoresed on 1% SDS-10% polyacrylamide discs (13). Gels were stained with Coomassie blue (8) and gel scans were done at 570 nm on a computerized Beckman spectrophotometer model DU-8 using the DU-8 gel scan module. Areas under the peaks were calculated by the same apparatus using the select mode. The main sources of error in the calculation of the relative abundance of the polypeptides lies in the measurement of peak area. The integrator needs a value for background subtraction which should be estimated by the operator. This is done by drawing a line from low areas of the electrophoretogram (9). Relative amounts of proteins in bands were taken as the area under each peak divided by total area of the electrophoretogram.

**Molecular Weight Determination.** Four mol wt markers (lysozyme, chymotrypsinogen A, ovalbumin, and BSA) from Mann Research Laboratories, New York, were electrophoresed separately and co-electrophoresed with wheat proteins. Their mol wt were 14,000, 24,000, 45,000, and 67,000, respectively. Molecular weights of wheat proteins were interpolated from these.

**Statistical Analyses.** Data from each polypeptide band were analyzed as a separate factorial experiment with seven cultivars, three treatments, and three representatives. Values of $F$ were calculated for cultivar, treatments, and cultivar $X$ treatments. Significance was taken as $P < 0.05$. Linear regression and simple linear correlation coefficients were used for evaluation of the relationship between protein and frost hardiness (9).

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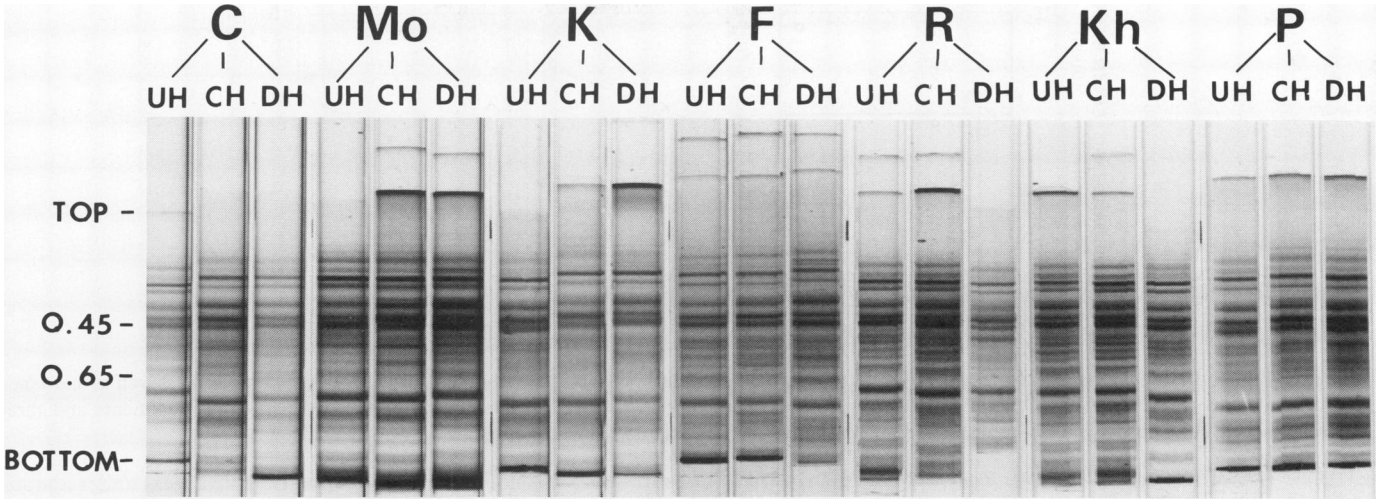


FIG. 1. Electrophoresis of soluble proteins from seven cultivars of winter wheat and rye. UH, unhardened; CH, cold conditioned; DH, desiccation stressed. Cultivars: Rye, Puma (P); wheat, Kharkov (Kh), Rideau (R), Fredrick (F), Kent (K), Monopol (Mo), Cappelle-Desprez (C).

## RESULTS

Detection of changes in the electrophoretic patterns of the soluble proteins following development of freezing tolerance in winter wheat and rye seedlings was carried out by comparing electrophoretograms from seedlings with various degree of freezing tolerance. To assess the degree of relationship between a polypeptidic band change on an electrophoretic gel and the freezing tolerance of the starting material, we postulated that the polypeptidic bands should obey the following criteria: (a) they must appear reproducibly and with the same intensity when protein extracts from similarly grown seedlings are electrophoresed. (b) Their intensity must increase or decrease proportionally to the degree of freezing tolerance of the starting material.

We therefore used the following experimental approach. Seedlings of contrasting degrees of freezing tolerance were obtained by growing different cultivars of winter wheat and rye in different environments. SDS-polyacrylamide gel electrophoresis patterns of the soluble protein extracts were compared. Band intensities were plotted *versus* the degree of freezing tolerance (as measured by $LD_{50}$ values) of the original seedlings. Correlation coefficients between polypeptidic band intensities and freezing tolerance must be significant to the 1% confidence level in order to indicate a relatively unequivocal role of the polypeptide.

Freezing tolerance developed in seedlings under cold acclimation or desiccation stress. The degrees of freezing tolerance was measured in epicotyls as their $LD_{50}$ values (Table I). Desiccation stress induced as much freezing tolerance as cold conditioning in epicotyls. The values of $LD_{50}$ summarized in Table I were used for the calculation of simple linear correlation coefficients of the protein-freezing tolerance relationship.

Table I. *Temperatures Required to Kill 50% ($LD_{50}$) of Epicotyls from Cold-Conditioned and Desiccation-Stressed Seedlings of Winter Wheat and Rye*

Seedlings were frozen to various temperatures and thawed. Epicotyls were severed and survival was measured by microscopy and regrowth.

| Cultivar | Unhardened | Cold Conditioned | Desiccation Stressed |
|---|---|---|---|
| | | °C | |
| Cappelle-Desprez | −4.5 | −8.5 | −9.5 |
| Monopol | −4.5 | −8.5 | −9.0 |
| Kent | −4.0 | −12 | −12 |
| Fredrick | −5.5 | −19 | −19 |
| Rideau | −5.5 | −22 | −21 |
| Kharkov | −5.0 | −37 | −31 |
| Puma | −6.5 | −61 | −61 |

Electrophoresis patterns of soluble proteins from seedling shoots of six cultivars of winter wheat and one of rye were compared. The amount of protein applied onto the gels varied from 50 to 250 μg. Eighteen protein bands were resolved by this system. Resolution varied from gel to gel owing to variation in the amount and volume of protein solution applied. Best resolution was obtained when 100 to 150 μg protein was applied in 15 μl solution. The 18 bands with $R_F$ values of 0.26, 0.29, 0.32, 0.35, 0.38, 0.43, 0.45, 0.49, 0.52, 0.57, 0.59, 0.62, 0.65, 0.68, 0.77, 0.84, 0.87, and 0.91 appeared consistently in all gels.

Cold conditioning and desiccation stress elicited many changes among the protein patterns of soluble proteins (Fig. 1). Most of the changes affected protein bands corresponding to medium electrophoretic mobility.

**Cultivar.** The amount of protein in 10 polypeptide bands with $R_F$ values of 0.38, 0.45, 0.52, 0.57, 0.59, 0.62, 0.65, 0.68, 0.77, and 0.84 differed significantly ($P < 0.05$) among the cultivars. The largest variation was observed for the band with $R_F$ of 0.45 between Kent and Puma. In the former cultivar, this polypeptide band made up to 7.3% of the total amount of soluble protein; in the latter it was 12%. The variation due to cultivar was not related to increased frost hardiness. It was probably due to genetic differences among the cultivars.

**Treatment.** For each treatment and each polypeptide band, the average amount of protein and a least significant difference were calculated (Table II). Bands with $R_F$ values of 0.43 and 0.77 increased significantly ($P < 0.05$) upon cold conditioning while those with $R_F$ values of 0.29, 0.38, 0.49, 0.52, 0.57, 0.59, and 0.62 increased upon desiccation stress. The band with an $R_F$ value of 0.45 was the only one to increase significantly upon both treatments. The results indicate that electrophoretic pattern changes are not the same in cold-conditioned as in desiccation-stressed seedlings.

**Cultivar X Treatment.** Amounts of protein in three polypeptide bands ($R_F$ values: 0.38, 0.45, and 0.65) differed significantly ($P < 0.05$) according to the $F$ values for cultivar $X$ treatments. Correlation coefficients between protein amounts and freezing tolerance were significant ($P < 0.01$) for two of these bands ($R_F$ values: 0.45 and 0.65). Protein amounts in these bands doubled in Puma rye seedlings when they were hardened by cold or by desiccation. Figure 2 illustrates the change of the amount of protein of the above cited bands in relation to decreasing $LD_{50}$ values. In this

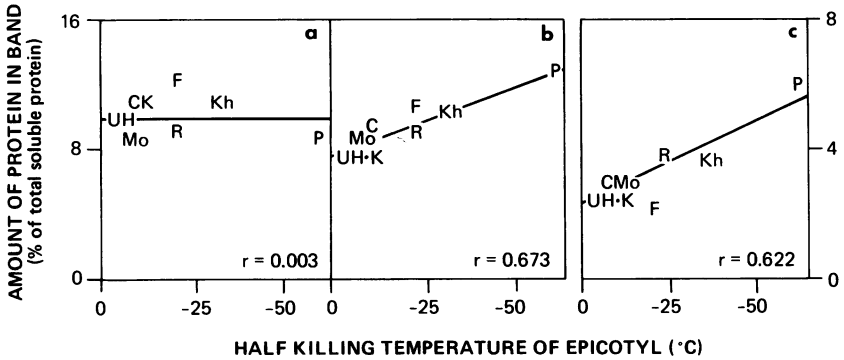


FIG. 2. Protein-freezing tolerance correlations for three polypeptide bands. UH is the amount of protein averaged over the seven unhardened cultivars and three representatives. Other symbols are the amounts of protein in cultivars averaged over the two hardening treatments and three representatives. Cultivar abbreviations as in Figure 1. Correlation coefficients and regression lines are shown.

Table II. *Dependence of Polypeptide Band Changes on Treatments*

Relative amounts of protein in polypeptide bands are percentages of total amounts of soluble protein. LSD are presented only for treatments differing significantly ($P < 0.05$).

| $R_F$ Values of Bands | Unhardened | Relative Amount of Soluble Protein | | LSD |
|---|---|---|---|---|
| | | Cold Conditioned | Desiccation Stressed | |
| | | % | | |
| 0.26 | 3.9 | 3.6 | 3.7 | |
| 0.29 | 3.4 | 3.7 | 4.3 | 0.7 |
| 0.32 | 5.7 | 6.3 | 5.7 | |
| 0.35 | 4.8 | 4.3 | 5.1 | |
| 0.38 | 9.0 | 9.2 | 11.5 | 1.3 |
| 0.43 | 7.5 | 10.6 | 7.6 | 1.6 |
| 0.45 | 7.8 | 10.2 | 10.3 | 1.3 |
| 0.49 | 5.2 | 5.9 | 6.4 | 0.9 |
| 0.52 | 4.6 | 4.9 | 6.0 | 0.7 |
| 0.57 | 4.4 | 4.7 | 5.9 | 0.8 |
| 0.59 | 3.7 | 4.3 | 5.0 | 0.7 |
| 0.62 | 4.0 | 4.0 | 5.4 | 0.6 |
| 0.65 | 2.7 | 3.4 | 3.3 | |
| 0.68 | 9.2 | 11.0 | 10.5 | |
| 0.77 | 8.6 | 10.7 | 9.8 | 1.5 |
| 0.84 | 3.7 | 4.6 | 3.9 | |
| 0.87 | 1.8 | 2.3 | 1.8 | |
| 0.91 | 2.5 | 3.0 | 3.4 | |

figure, we compared the amount of protein in hardened cultivars (by cold or by desiccation indistinctively) to that in unhardened plants. Increase of the former over the latter was proportional to $LD_{50}$ in Figure 2, b and c, but not in Figure 2a.

## DISCUSSION

The SDS-polyacrylamide gel system we used does allow separation of the proteins according to their apparent mol wt. Changes in the electrophoretic patterns following hardening should therefore reflect variations in the relative abundance of polypeptides with possible splitting of some. Biochemical modifications on the polypeptides such as methylation, phosphorylation, or conformational changes may escape detection in our system because they do not alter significantly the mol wt. Detection of such changes was not desirable because, although protein alterations are probably occurring in the hardening process, they are also part of the normal metabolism of the cell. Changes in the mol wt instead reflect synthesis or cleavage of proteins and is easier to interpret in terms of appearance of cryoprotective proteins. No attempt was made to minimize the action of proteinases. Use of proteinase inhibitors was not thought to be fully reliable unless under a much more complicated extraction procedure. Cleavage of polypeptides during extraction procedure and handling of samples results in loss of information about the protein-frost tolerance relationship. However, the remaining information remains valuable as a minimal estimation of the changes.

There were significant differences in the abundance of 13 polypeptide bands of the 18 we analyzed. The variation due to cultivar probably originates from genetic differences between the cultivars. Electrophoretic pattern changes due to treatments were as numerous as those due to cultivars affecting 10 bands. Relative abundance of the polypeptides with $R_F$ values of 0.29, 0.38, 0.43, 0.52, 0.57, and 0.62 did not change the same manner in cold-conditioned and desiccation-stressed seedlings despite the fact that their freezing tolerance increased the same amount. The two treatments are thus nonequivalent although these observations do not imply that development of freezing tolerance does not proceed by a similar mechanism under the two conditions.

In three bands ($R_F = 0.38$, 0.45, and 0.65), a significant variation ($P < 0.05$) due to cultivar $X$ treatment was observed. This result can be interpreted as a different response of some cultivars to some treatment. Significant protein-$LD_{50}$ relationships had to be found among these three bands. Linear correlations were found very significant for two of these ($R_F = 0.45$ and 0.65). These polypeptide bands corresponding to apparent mol wt of 46,000 and 30,000 may play a role in the development of freezing tolerance. However, they represent no more than a 2-fold increase of polypeptides already present in unhardened plants. On this basis, it is not possible to assign them a cryoprotective role with confidence. It seems more reasonable that these bands may subdivide into many polypeptides, one of which could have a cryoprotective role or could have an enzymic activity important for the hardy cell.

We did not detect the appearance of any new polypeptide or the striking increase of any one upon hardening. The changes in the electrophoretic patterns were small on the average. Our results are similar to those of Faw and co-workers (9, 10) who found increases in soluble protein amounts with no striking electrophoretic pattern change in response to cold hardening of alfalfa. These facts along with the presence of different polypeptidic changes in cold-conditioned and desiccation-stressed seedling lead me to these conclusions: (a) two polypeptide band changes are correlated with increased freezing tolerance. These bands do not represent individual polypeptides to which one could assign a cryoprotective role but they may contain such polypeptides. (b) Cold conditioning and desiccation stress are not equivalent but a common mechanism for the development of freezing tolerance under the two conditions cannot be excluded.

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