Differences in Activities Related to Cessation of Synthesis of Inducible Proteins as an Early Response to Cold between Hardy and Less Hardy Cultivars of Winter Wheat

Yusuke Matsuda, Tohru Okuda, and Shonosuke Sagisaka

The Institute of Low Temperature Science, Hokkaido University, Sapporo 060, Japan

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Using two sets of cultivars, two hardy and two less hardy ones, we studied differences in the regulation of the synthesis of cold-inducible proteins at an early stage of cold acclimation. During 11 days of cold treatment, all four cultivars had changes in rates of protein synthesis, which were divided into four phases. The difference in the regulation of synthesis of the protein was evident at the third and fourth phases. In the less hardy cultivars, rates of synthesis of cold-inducible proteins started to decline and the profiles of proteins being synthesized resembled those of control samples (without cold treatment). In hardy cultivars, rates of synthesis of typical cold-inducible proteins remained unchanged, suggesting that some regulatory mechanisms regulate the continued cold-inducible synthesis of proteins in the latter cultivars by acting, presumably, at particular target sites within the crown cells.

Analysis of freezing tolerance showed that in stems and crowns of winter wheat, the cold-inducible proteins do not contribute directly to the freezing tolerance, while some may have roles in bringing about increased longevity in cold environments.

In an earlier report, we described changes in activities related to protein metabolism during an early stage of cold acclimation in winter wheat. In the crown of cv. Horoshirikomugi, which is a typical hardy cultivar of winter wheat, protein synthetic activities were found to be divided into four phases, and the basic profiles of changes in activities were as follows. During the lag period (phase 1), mRNAs inducible by cold treatment are not synthesized, and during his first phase, translation of pre-existing mRNAs is not depressed. During phases 2 and 3, newly induced mRNAs, together with some of pre-existing mRNAs are translated preferentially and then rates of synthesis of some of them start to decrease to an appreciable extent. During the early stages of acclimation, after the synthesis of inducible mRNAs, translation of some pre-existing mRNAs is efficiently depressed in vivo. When mRNAs are isolated at phases 2, 3, and 4 and translated in vitro, the resultant autoradiograms are indistinguishable from one another (Matsuda et al., unpublished observation). These results suggest that, to sustain plant life under show or in frozen fields, an obligatory metabolic shift occurs during the early stages of cold acclimation in winter wheat.

Among cultivars of winter wheat, cv. Horoshirikomugi is a representative of hardy cultivars, and there are a number of less hardy cultivars. Previous studies showed that conspicuously high rates of consumption of stored carbohydrates (about 6 times the rate in hardy cultivars) in less hardy cultivars is one of the fundamental differences between the two types. Futil events, such as growth under show, that occur in less hardy cultivars result in the waste of stored carbohydrates and, accordingly, in the shortening of the lifespan of these plants under stressed conditions. Given the low rate of consumption of carbohydrates by hardy cultivars, we presume that some strict system of metabolic regulation is operative. Their metabolic ability to adjust efficiently to cold environments may involve regulation of protein-synthetic activities at the early stage of environmental changes.

During a period of cold acclimation, the increase in freezing tolerance and ability to survive for a long period during wintering (cold hardiness) is obligatory for cold-tolerant plants, such as winter wheat. Changes in lipid composition and increases in sugar levels and protein concentration have been reported in relation to cold acclimation. Cold treatment of cold-tolerant plants generated "cold-regulated polypeptides" in, for example, alfalfa, Arabidopsis, wheat, and Brassica. Among the polypeptides found in these plants, the "boiling stable protein," was isolated as a typical protein from leaves of winter wheat, Triticum aestivum L. cv. Winoka, and Arabidopsis thaliana L. cv. Landsberg erecta. The structural and biochemical changes that take place in plant cells during cold acclimation may have something to do with the function of the "boiling stable protein." In winter wheat, the stems and crowns are the most important tissues for regrowth after the snow melts, but we are unable to detect the "boiling stable protein" in crowns of winter wheat. Therefore, whether any cold-regulated protein that contributes significantly to freezing tolerance in winter wheat in vivo remains to be discovered. Since nothing is known about protein-synthetic activities that are linked to cold hardness, comparisons were done using hardy and less hardy cultivars of winter wheat, and the results of the studies are presented in this paper.

Materials and Methods

Chemicals. L-[35S]-Methionine was purchased from American Radio-labeled Chemicals Inc. (St. Louis, MO, U.S.A.), Pharmalytes were from Pharmacia LKB (Uppsala, Sweden), and a silver-staining kit (2-D-silver

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* To whom correspondence should be addressed.

Abbreviations: IEF, isoelectric focusing; LTso, temperature for 50% lethality.
stain) was from Daiichikagaku Inc. (Tokyo, Japan). All other chemicals were of analytical grade. X-Ray films (Fuji-RX film) were from Fuji Film Inc. (Kanagawa, Japan).

**Plant materials.** Seeds of winter wheat (*Triticum aestivum* L.) were kindly provided by Drs. S. Ozeki and T. Kuwabara of the Hokkaido National Agricultural Experiment Station. Cultivars used were two hardy ones, namely cv. Horoshirikomugi (cv. HOK) and cv. Mukakomugi (cv. MKA) and two less hardy ones, namely cv. Norin 61 (cv. N61) and cv. Shiroganekomugi (cv. SRG). The growth conditions were the same as described previously. In brief, seeds were allowed to germinate on moist vermiculite for 4 d in darkness at 28 °C. Then seedlings were cultivated under artificial light (20,000 lux, 16-h photoperiod) at 24 to 28 °C for 7 d (control), and finally they were put into wintering conditions (0 °C in darkness). Cold treatments were done for 3 and 11 d.

**Radiolabelling of proteins in vivo.** Ten crowns (about 20 mg) were excised from seedlings for each treatment immediately before incubation, and they were incubated in a mixture of 10 mM Tris-HCl, pH 7.0, 20 mM KCl, and 150 μCi/ml of L-[35S]-methionine (1.082 Ci/mmol). Incubations were done for 8 h at 28 °C for controls and for 20 h at 0 °C for test samples.

**Extraction, analysis, and fluorography of radiolabelled proteins.** Incorporation of the radiolabel into proteins was stopped by rinsing the crowns with a 4 mM solution of unlabelled dl-methionine. Then, crowns were washed with a solution of 0.1 M NaCl and 0.1% Triton X-100 and then with distilled water. Homogenization of the tissue and extraction of proteins were done as described previously. In brief, the tissues were ground in 200 μl of phenol that had been saturated with buffer A (120 mM Tris-HCl, pH 7.5, 50 mM EDTA, 100 mM KCl, 2% mercaptoethanol, and 2% SDS). To the homogenate, 200 μl of buffer A were added, the mixture was vortexed briefly, and then it was centrifuged at 10,000 × g for 10 sec. The phenol phase was washed once with buffer A, mixed with 5 volumes of 100 mM ammonium acetate with 1% 2-mercaptoethanol in methanol (at −20 °C), and then left for 2 h at −85 °C. Proteins were pelleted by centrifugation at 15,000 × g for 10 min, washed twice with 100 mM ammonium acetate in methanol, and then with acetone that contained 1% 2-mercaptoethanol. After drying, proteins were dissolved in 50 μl of O’Farrell’s lysis buffer and separated by a modified version of the two-dimensional PAGE technique of O’Farrell, using Pharmalytes pH 3.0 to 10.0 and pH 5.0 to 8.0 (1:4 w/v) at a final concentration of 3%. First-dimensional IEF was done under the following constant voltages: 400 V for 2 h; 600 V for 3 h; 800 V for 3 h; and 1000 V for 1 h. The duration of fluorography was calculated as follows: duration (days) = 2.5 × 10^6/total applied radioactivity (cpm) of each sample. After completion of fluorography, gels were reswollen in distilled water and stained with silver.

**Measurement of freezing tolerance.** Seedlings of control plants and seedlings incubated at 0 °C for 3 d, 5 d, 8 d, and 11 d were removed from moist vermiculite. Ten to twenty seedlings were placed in a 50-ml plastic tube together with a moist Kimwipe and covered with Saran wrap (Asahi Kasei Kogyo Inc, Tokyo, Japan) to prevent desiccation of the seedlings. To avoid super cooling of extracellular water, 2 to 3 small pieces of ice were placed on the surface of the seedlings. Seedlings were then pre-equilibrated at −2.5 °C and cooled at a rate of 1.5 °C/h to successively lower temperatures. At selected temperatures, they were taken out from the freezer. Then they were placed at 0 °C for 1 d and transferred to a growth chamber (24 to 28 °C, 20,000 lux, 16-h photoperiod). Freezing tolerance of seedlings was assessed in terms of the ability to reinitiate growth over the course of more than 1 wk and ratios of surviving seedlings.

![Fig. 1. Comparison of Protein-synthetic Activities in Crowns of Four Cultivars of Winter Wheat without Cold Treatment.](image-url)

N-HOK, cv. Horoshirikomugi without cold treatment; N-MKA, cv. Mukakomugi without cold treatment; N-N61, cv. Norin 61 without cold treatment; N-SRG, cv. Shiroganekomugi without cold treatment. The excised crowns were incubated with 150 μCi/ml L-[35S]-methionine (1082 Ci/mmol) for 8 h at 28 °C. Arrowheads indicate cold-inducible proteins (C1 and C2). The positions of protein standards are indicated as M, × 10^5. Other conditions are described in the text.
were calculated.

Results
Responses to cold treatment: the first phase
At 28°C, the two sets of cultivars, the hardy ones, (cv. HOK and cv. MKA) and the less hardy ones, (cv. N61 and cv. SRG), respectively, gave almost the same patterns of protein synthesis except that low levels of proteins C1 and C2 were synthesized in the less hardy cultivars, N61 and SRG (Fig. 1). The three cultivars, cv. MKA, cv. N61, and cv. SRG, had the same lag times for protein synthesis as cv. HOK did in response to cold treatment in previous experiments. There was no formation of inducible proteins nor any indication of a decrease in levels of pre-existing proteins during this period (data not shown).

Improved resolution of radiolabelled proteins by the modified PAGE procedures
In a previous report, proteins referred to as C1, C2, C10, C12a, and C12b were described as proteins that were inducible by cold treatment of cv. HOK. In these studies, the modified version of the two-dimensional PAGE technique of O'Farrell resulted in resolution of C10 into C10a and C10b, and of C12 proteins into C12a, C12b, and C12c (Figs. 2 and 3, W-HOK and W-MKA). The modified procedure made it possible to compare the inducible synthesis of certain proteins between the hardy and less hardy cultivars.

Responses to cold during the third phase
In the case of cv. HOK, after cold treatment for a period from 12 to 24h, synthesis of inducible proteins began, together with reinitiation or depression of the rate of synthesis of pre-existing proteins (2nd phase), and the synthetic activities reached a maximum between two and five days of cold treatment (3rd phase). The other three cultivars, cv. MKA, cv. N61, and cv. SRG, showed the same responses with time to cold treatment during the third phase; the cold-inducible proteins were synthesized very actively (Fig. 2, arrowheads) and rates of synthesis of some pre-existing proteins declined (Fig. 2, squares). However, there was a prominent difference in the rates of synthesis of proteins in the C12 group between the two sets of cultivars. The fluorograms in Fig. 2 show that the amount of the radiolabel in spot C12a is similar in the four cultivars, while in spot C12b the amount of radiolabel in the less

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Fig. 2. Comparison of Protein-synthetic Activities in Crowns of Four Cultivars of Winter Wheat after Cold Treatment for 3 Days. W-HOK3, cv. Horoshirimokumugi after cold treatment for 3d; W-MKA3, cv. Mukakomugi after cold treatment for 3d; N613, cv. Norin 61 after cold treatment for 3d; W-SRG3, cv. Shiroganekeumugi after cold treatment for 3d. The excised crowns were incubated with 150 μCi/ml L-[35S]-methionine (1082 Ci/mmol) for 20h at 0°C. Arrowheads numbered C1 to C3, C8, C10a, C10b, C12a to C12c, and C21 indicate cold-inducible proteins; arrowheads numbered C6a to C6c indicate proteins whose synthesis was stimulated by cold; squares indicate pre-existing proteins with transiently decreased rates of synthesis in crowns of cv. Horoshirimokumugi between 1d and 5d of cold treatment. The positions of protein standards are indicated as M, x 10^3. Other conditions are described in the text.
hardy cultivars is clearly lower than in the hardy ones. The fluorograms also show that spots C12c and C3 are missing in the less hardy cultivars, N61 and SRG.

Other spots, such as c6a, c6b, and c6c, which represent pre-existing proteins the synthesis of which was stimulated by cold treatment, were heavily labeled at this phase in all four cultivars. Temporal profiles of the synthesis of these proteins were similar in the hardy and less hardy cultivars. There were no differences in spots C8, C10a, and C10b between cultivars during the third phase.

Responses during the fourth phase

After 11 d of cold treatment, the two hardy cultivars gave almost the same patterns of distribution of radion-labelled proteins (Fig. 3, W-HOK11 and W-MKA11) as seen during the third phase (and also as observed earlier) with the exception that synthesis of the same preexisting proteins was reinitiated (Fig. 3, squares). By contrast, in the two less hardy cultivars, the amount of label in cold-inducible proteins, such as C1, C2, and C10b, started to decrease (Fig. 3, W-N611 and W-SRG11, arrowheads numbered C1, C2, and C16b). In the C12 group, the radiolabel in C12c seemed to be missing or was very hard to detect, and synthesis of C12b was depressed still more than during the third phase (Fig. 3, W-N611 and W-SRG11, arrowheads numbered C12b and C12c). Moreover, in the less hardy cultivars, synthesis of proteins other than cold-inducible proteins was stimulated during this phase (Fig. 3, W-N6111 and W-SRG11, spots surrounded by broken squares).

Steady protein-synthetic activities at an early stage of cold treatment

There was no serious change in the rates of synthesis (incorporation of radiolabel) of proteins during the third and fourth phases (Table I) in the respective cultivars. Therefore, an ability to synthesize proteins found to be kept in similar levels in all four cultivars even after 10 d of cold treatment.

Changes in freezing tolerance of four cultivars during cold treatment

As shown in Table II, freezing tolerance of the four cultivars reached a maximum after 8 d of cold treatment. Differences in the rapidity of acquisition of freezing tolerance and levels of freezing tolerance did not necessarily correspond to differences in wintering abilities among cultivars. Two cultivars, HOK and SRG, showed an in-
crease in freezing tolerance after 3 d of cold treatment, but after at least 5 d of cold treatment an increase in freezing tolerance was also observed in the other two cultivars, N61 and MKA. SRG, one of the less cold-tolerant cultivars had the same freezing tolerance, with an LT°s of about −7°C, as cv. HOK and cv. MKA after cold treatment for 8 d. After 11 d of cold treatment, freezing tolerance of all four cultivars declined rapidly.

**Table I.** Incorporation of L-[35S] Methionine into Crown Proteins of Four Cultivars of Winter Wheat

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Specific radioactivity (cpm/μg protein)</th>
<th>Duration of cold treatment (d)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0°C (control)</td>
</tr>
<tr>
<td>HOK</td>
<td>10,530</td>
<td>5100</td>
</tr>
<tr>
<td>MKA</td>
<td>13,380</td>
<td>6770</td>
</tr>
<tr>
<td>N61</td>
<td>16,810</td>
<td>6290</td>
</tr>
<tr>
<td>SRG</td>
<td>13,680</td>
<td>7440</td>
</tr>
</tbody>
</table>


ootnote{a} Incubated for 8 h at 28°C.

ootnote{b} Incubated for 20 h at 0°C.

**Table II.** Survival of Seedlings of Four Cultivars of Winter Wheat after Freezing at Various Temperatures

<table>
<thead>
<tr>
<th>Duration of cold treatment (d)</th>
<th>Cultivar</th>
<th>Percentage of surviving plants (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>−4</td>
</tr>
<tr>
<td>3</td>
<td>HOK</td>
<td>96.6</td>
</tr>
<tr>
<td></td>
<td>MKA</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>N61</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>SRG</td>
<td>100.0</td>
</tr>
<tr>
<td>5</td>
<td>HOK</td>
<td>97.0</td>
</tr>
<tr>
<td></td>
<td>MKA</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>N61</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>SRG</td>
<td>100.0</td>
</tr>
<tr>
<td>8</td>
<td>HOK</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>MKA</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>N61</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>SRG</td>
<td>100.0</td>
</tr>
<tr>
<td>11</td>
<td>HOK</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>MKA</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>N61</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>SRG</td>
<td>100.0</td>
</tr>
</tbody>
</table>


**Discussion**

In terms of the general features of the changes in rates of synthesis of inducible proteins, the hardy and less hardy cultivars of winter wheat had different responses to cold treatments. In addition to differences in numbers of cold-inducible proteins (Figs. 2 and 3, proteins numbered C3 and C12c, for example), rates of synthesis of cold-inducible proteins declined earlier in the less hardy cultivars (N61 and SRG), but in the hardy ones (HOK and MKA), the activities remained fairly constant (Fig. 3, proteins numbered C1, C2, C10b, and C12b). The functions of these inducible proteins are not yet known, but they may be involved in long-term wintering.

Previous reports showed that one of the major differences between the hardy (HOK) and less hardy (N61) cultivars of winter wheat seemed to result from a difference in basal metabolic rate as it related to consumption of carbohydrate reserves under snow. In the less hardy cultivar (N61), the level of the reserves decreased rapidly and, when the stored hexose fell to a level below 80 μmol g⁻¹ fresh weight, the plant was no longer able to initiate regrowth in early spring. Furthermore, it was shown that N61 continued to grow under unfavorably low temperatures under snow. In contrast, tissues of HOK accumulated nearly the same levels of carbohydrate reserves as those in N61 in the fall and, since the reserves decreased slowly (1.3 to 1.7 μmol g⁻¹ fresh weight in the stem and crown of HOK during the period from February 16 to March 16), the level only reached a critical value by late July under simulated wintering conditions in ice. Since the difference in freezing tolerance between the two sets of cultivars does not directly affect their wintering ability under snow and since the levels of reserved carbohydrates are directly related to the

**Fig. 4.** Silver-staining Analysis of the Accumulation of Cold-inducible Proteins C10a, C10b, and the C12 Group in Crowns of cv. Horoshirikomugi during Cold Treatment.

Proteins in crowns of cv. Horoshirikomugi, treated by incubation in the cold for 3 d, 5 d, 7 d, 9 d, 11 d, and 13 d were extracted, separated by two-dimensional PAGE, and stained with silver. Symbols are explained in the legend to Fig. 2. Other conditions are described in the text.
longevity of the winter wheat, some of the inducible proteins may play a role in inhibiting futile reactions so as to reduce the consumption of hexose. Other possibilities for the function of these proteins included inhibition of growth under snow, which results in the waste of reserved carbohydrates.

This study found that, even in the presence of most of the cold-inducible proteins, the seedlings failed to have maximum freezing tolerance (Table II). The seedlings have appropriate enzymatic activities for adaptation to low-temperature environments but seem to consume considerable amounts of stored carbohydrates to withstand more than 10 days under low-temperature conditions in darkness. Therefore, even in the presence of the major fraction of cold-inducible proteins, decreases in the reserve levels of carbohydrates result in a decrease in freezing tolerance. This result is in accord with our hypothesis that the fundamental character of freezing tolerance or cold hardiness of higher plants is determined neither by an enzyme, by a single protein, or by a single substrate, but results from the sum of the functions of cellular constituents or metabolic activities, such as peroxide-scavenging systems. The contribution of some constituents to the tolerance may be somewhat variable, being dependent on the growth phase. After the predominant induction of cold-inducible proteins, the hardy cultivars of winter wheat continue to adjust their regulatory systems to the new environmental conditions. However, in the less hardy cultivars, the regulatory systems that control protein synthesis may exert their effects by turning off the synthesis of the cold-inducible proteins, and the characteristics of the synthesis of proteins would return to those before the cold treatment. Comparison of Fig. 1 (before cold treatment) and 3 (during the fourth phase) clearly indicates that changes in protein profiles between the cold-tolerant and less tolerant cultivars emerged during the fourth phase, in addition to intrinsic differences in rates of synthesis of C10b, C12b, and C12c (Fig. 3, compare HOK and MKA to N61 and SRG).

References