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COLD-INDUCED ACCUMULATION OF PROTEIN IN THE LEAVES OF SPRING AND WINTER BARLEY CULTIVARS

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Electrophoretic pattern and quantitative changes in soluble proteins were determined in the leaves of spring and winter cultivars of barley (*Hordeum vulgare* L., cv. Makouei and cv. Reyhan, respectively) exposed to 4 °C for 14 d. Seedlings were grown in a controlled growth chamber for 2 weeks at a constant air temperature of 20 °C and then transferred to constant 4 °C for 14 d followed by returning to 20 °C (cold treatment), or they were maintained throughout at 20 °C during the experimental period of 40 d (control treatment).

Plants were sampled every 48 h for leaf fresh weight measurements. Total leaf soluble proteins were extracted and their concentration was either determined by a colorimetric method, or size-fractionated on SDS-PAGE. Low temperature-induced increases in protein amount occurred over the second week of exposure to cold treatment irrespective of cultivar: the winter cultivar was 2 d prior in this response. The protein patterns and their density showed differences between-cultivars and between-temperature treatments. A new cold-induced polypeptide was recognized in the leaves of winter barley cultivar on day 22 (8 d at 4 °C) compared to the control. This polypeptide was produced earlier over the first 48 h of low temperature in the winter cultivar compared with the spring one, recognizing in the leaves of cold-treated seedling until day 26. This more rapid response to a low temperature by the winter barley cultivar indicates a more sensitive response compared with the spring barley, probably cold-shock protein is a component of this cold-induced response.

Keywords: Hordeum vulgare L. – barley – leaf protein – cold – SDS-PAGE – Bradford assay

INTRODUCTION

Suboptimal temperature is one of the primary stresses limiting growth, productivity and distribution of plant [2]. Frost resistance in barley is an important agronomic character because its growing has been shifting for a number of years from spring to autumn sowing as winter varieties are higher-yielding than spring cultivars. The tolerance of winter cereal to low temperatures depends on the physiological process known as hardening or acclimation that occurs when plants are exposed to temperatures ranging from 0 to 5 °C [22, 23, 35]. In some plant species, one feature of cold

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hardening is an accumulation in soluble protein content e.g. in alfalfa *(Medicago sativa)* [32], in wheat *(Triticum aestivum)* and rye *(Secale cereale)* [6, 22, 23, 26, 27, 41], in barley *(Hordeum vulgare)* [4, 5, 8, 9, 10, 11] and in canola *(Brassica napus)* [24, 28, 38, 40]. For example in wheat, the accumulation of a high molecular weight of 200 kDa [22, 36, 41] and 310 kDa [26] polypeptide is a major change that occurred during hardening. The latter cold stress protein was also reported in rye [27]. Recently, Thomashow [45] reviewed genes induced during cold acclimation encoding different proteins in several plants e.g. *Arabidopsis,* canola, barley, alfalfa, spinach and wheat.

Cold acclimation involves a number of biochemical, physiological and molecular changes that enable certain plant species to withstand periods of very intense cold [5, 22, 23, 29, 35, 43]. Cellular and metabolic changes that occur during cold acclimation include e.g. variation in the membrane properties and composition, increase of enzyme activities and accumulation of specific cold regulated (COR) proteins [5, 8, 9, 10, 11]. Synthesis of specific proteins seems to be an important mechanism involved in increasing freezing tolerance during cold acclimation [1, 10, 16, 17, 18, 21, 41, 42]. The 14 kDa cold-regulated protein (COR14) is a polypeptide accumulated under low temperature conditions in the chloroplasts of barley leaves [11]. The corresponding gene, cor14b (formerly *pt59*) [9], is expressed in the leaves of barley and of the related cereals, during plant exposure to low temperatures and was found to be regulated by light. COR75 is the predominant COR protein in barley, induced by low temperature (4 °C). The hardening-triggered accumulation of a barley cold-regulated protein of 75 kDa (COR75) has been investigated in several genotypes featuring contrasting frost tolerance capacity [34, 37]. COR75 is accumulated faster during the first days at low temperatures in frost resistant varieties when compared with extremely sensitive varieties, nevertheless, after 7 d of hardening, COR75 is expressed at the same level in all genotypes tested despite their differences in frost tolerance. Yet, the extend of frost resistance of a given genotype was not found to be related to the amount of COR75 accumulated in the tissues. The expression pattern of COR75 in response to various environmental stresses was monitored in plant at the first-leaf stage [34, 37]. Besides low temperature, COR75 can also be induced by ABA or drought stress indicating that this protein may be a general response to variation in tissue moisture status [10]. After purification and N-terminal microsequence, this protein (COR75) was found to be DHN5 [5]. The appearance of high molecular weight DHNs (dehydrins) has been correlated with cold tolerance acquisition in wheat and barley [4]. They reported the accumulation of an 80-kDa DHN-like protein (P-80) in barley under cold acclimation 6/4 °C (day/night) in cold-acclimated leaves [4].

In this study, we examined whether spring and winter cultivars of barley responded differently to cold treatment through differential protein accumulation. The aim of the work reported here was to test this hypothesis by comparing quantitative amounts and electrophoretic patterns of the total soluble proteins accumulation in the leaves of spring and winter cultivars of barley in a treatment comprising shifts from 20 to 4 back to 20 °C.

MATERIALS AND METHODS

Plant material and growth conditions

Caryopses of a spring (cv. Reyhan) and a winter (cv. Makouei) barley (*Hordeum vulgare* L.) cultivars supplied from The Seed and Plant Improvement Institute (SPII), Karaj, Iran, were initially grown in plastic pots (150 mm diameter × 150 mm deep) filled with a mixture of five parts soft mold leaves and two parts loamy sand. The Reyhan cultivar with spring habit is susceptible to cold and semi-resistant to powdery mildew and tolerant to barley stripe disease, growing in temperate and semi-aried regions of Iran. Makouei, a winter habit cultivar, is resistant to cold and powdery mildew and tolerant to barley stripe disease. This variety is cultivated in elevated regions in cold winter season of the country.

The experiment was realized in a complete randomized design with three replications. Each replication consisted of two pots of five seedlings per cultivar, temperature treatment and sampling time combinations. The pots were kept in a controlled growth chamber at a constant air temperature of 20 ± 1 °C with illumination provided by white fluorescent tubes at a fluence rate of 140 W m^{-2} PAR at soil level for 12 h d^{-1} (0800–2000 h). Seedlings were maintained in these conditions until experimental day 14. Note that the day of sowing was designated as experimental day 0 (Fig. 1). On day 14, after taking a sample, the seedlings were remained at a constant 20 °C throughout the rest of 40 days of experimental period (control treatment) or transferred to cold growth chamber at a constant air temperature of 4 ± 1 °C at the same fluence rate and photoperiod as above for 14 d. These cold-treated seedlings were then returned to 20 °C on day 28 until the end of the experiment (day 40; cold treatment).

Sampling times

Over the experimental period (days 14–40), 3 × 0.5 g of total leaf fresh weight samples [8, 12, 13, 24] were sampled randomly every 48 h from two pots of five seedlings. In other words, at the end of experiment, the sampling times for each cultivar-low temperature treatment combination were days 14 (before transfer to 4 °C), 16, 18, 20, 22, 24, 26, 28 (during exposure to 4 °C), 30, 32, 34, 36, 38 and 40 (after the return of cold-treated seedlings to 20 °C; Fig. 1). Hence, in total, 14 sampling times were considered in this experiment. At the same sampling times and daytime, samples were also taken from the controls.

Protein extraction

Total soluble proteins were extracted from the leaves with a modification of the method described by Guy et al. [19]. This consisted of homogenization with a chilled mortar and pestle using a buffer containing ice-cold 50 mM Tris-HCl, pH 7.5; 2 mM

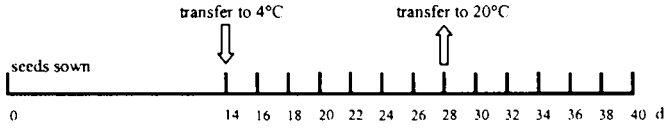


Fig. 1. Experimental protocol. The arrows indicate the days of the downshift and upshift of temperatures, and sampling days

EDTA and 0.04% (v/v) 2-mercaptoethanol. The homogenate was centrifuged at 4000 rpm for 30 min at room temperature [31]. Supernatant was re-centrifuged for 20 min and stored at –20 °C for later analysis [20].

Quantitation of protein using the Bradford assay

Protein extracts were thawed and their concentration was determined by a calorimetric method based on that described by Bradford [3], using a commercially available reagent (Bio-Rad protein assay dye reagent). In the Bradford assay, protein concentration is determined by quantitating the binding of the dye, Coomassie Brilliant Blue G-250, to the unknown protein solution, as compared to known standards. Tubes containing 100 µl aliquots of known concentrations of Bovine Serum Albumin (BSA; 0.156 mg 1^{-1} to 10 mg 1^{-1} in 0.15 M NaCl), were prepared. Blank tubes containing 100 µl of 0.15 M NaCl were also prepared. One ml Coomassie Brilliant Blue solution was added to each tube and the mixtures vortexed. The reactions were left at room temperature for 2 min. The absorbance at wavelength of 595 nm was determined against the blank and the standard curve of absorbance versus protein concentration plotted [7]. Reactions containing dilutions of the soluble protein extracts (unknown concentrations) were set up as above and the absorbance at 595 nm determined. The proteins concentration of the extracts was determined form the standard curve, using an Unicam 8620 UV/VIS (USA) Spectrophotometer.

Protein electrophoresis

The leaf soluble proteins homogenate was mixed with buffer containing 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 5% (v/v) 2-mercaptoethanol, 10% glycerol and 0.01% bromophenol blue and then heated at 100 °C for 2 min and centrifuged at 4000 rpm for 30 min. Supernatant was stored at –20 °C for later analysis. Protein extracts were thawed and separated, as polypeptides, by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% gels: 10 µg of soluble total protein was loaded in each well. Gels were fixed in trichloroacetic acid and stained in [0.1% (w/v) coomassie brilliant blue G-250, 10% (v/v) glacial acetic acid and 40% (v/v) methanol]. The gels were placed in fresh prepared destaining solution (40% methanol, 10% glacial acetic acid)

with gentle shaking for 30 min. This process was repeated until the gels were sufficiently destained. The gels were then photographed and/or stored in 10% glacial acetic acid.

Statistical analysis

The quantitative amounts of total leaf soluble proteins were statistically analyzed using three-factorial balanced analysis of variance (ANOVA) on the basis of randomized complete design (RCD) with three replications. Cultivars, temperature treatments and sampling times were considered as factors with 2, 2 and 14 levels, respectively. The protein data were analyzed after log x+1 transformation. Analysis of variance was conducted using Multi-Factorial Balanced Model in MINITAB Statistical Software (Minitab Inc., State College, PA, USA) [15, 39]; identification of differences in response of the cultivars to the temperature treatments at the sampling times are based on the outcome of these tests. An additional analysis was carried out in order to determine the interaction between temperature treatments and sampling time for each cultivar separately [33, 46].

RESULTS

Leaf total soluble proteins increase in both cultivars at 4 °C

In the analysis of variance (Table 1), the cultivar, temperature treatment, sampling time and temperature treatment × sampling time interaction were highly significant ($P < 0.001$), as was the cultivar × temperature treatment × sampling time interaction

Table 1
Mean squares (MS) of the ANOVA for leaf soluble proteins of barley (*Hordeum vulgare* L.) cultivars

Source of variation	df	MS
Cultivar (cv.)	1	1.47308***
Temperature treatment (T)	1	0.68580***
Sampling time (S)	13	0.32391***
cv. × T	1	0.04788
cv. × S	13	0.04993
T × S	13	0.49631***
cv. × T × S	13	0.08950**
Error	112	0.03861
Total	167	

**Significant at P = 0.01.
***Significant at P = 0.001.

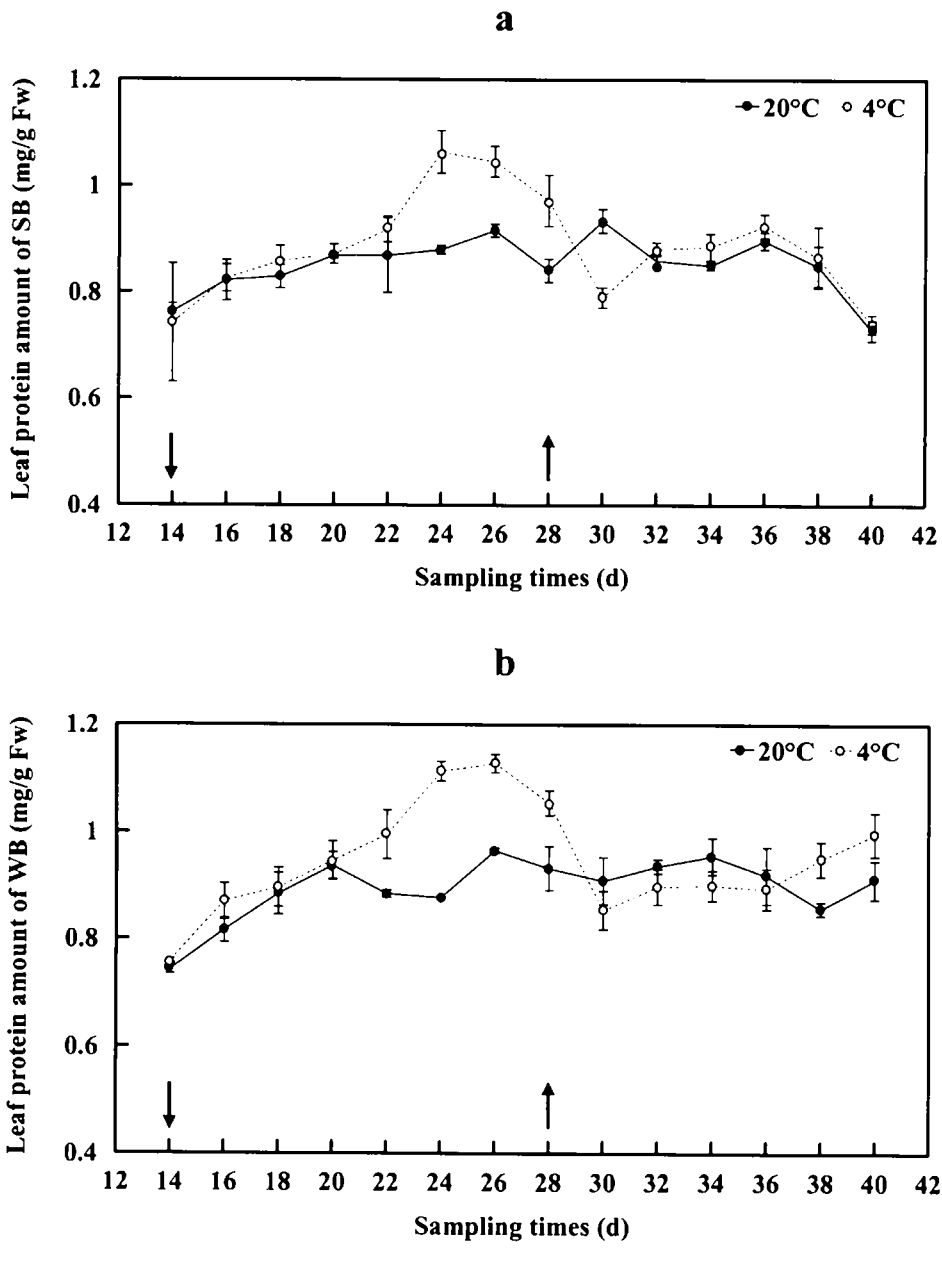


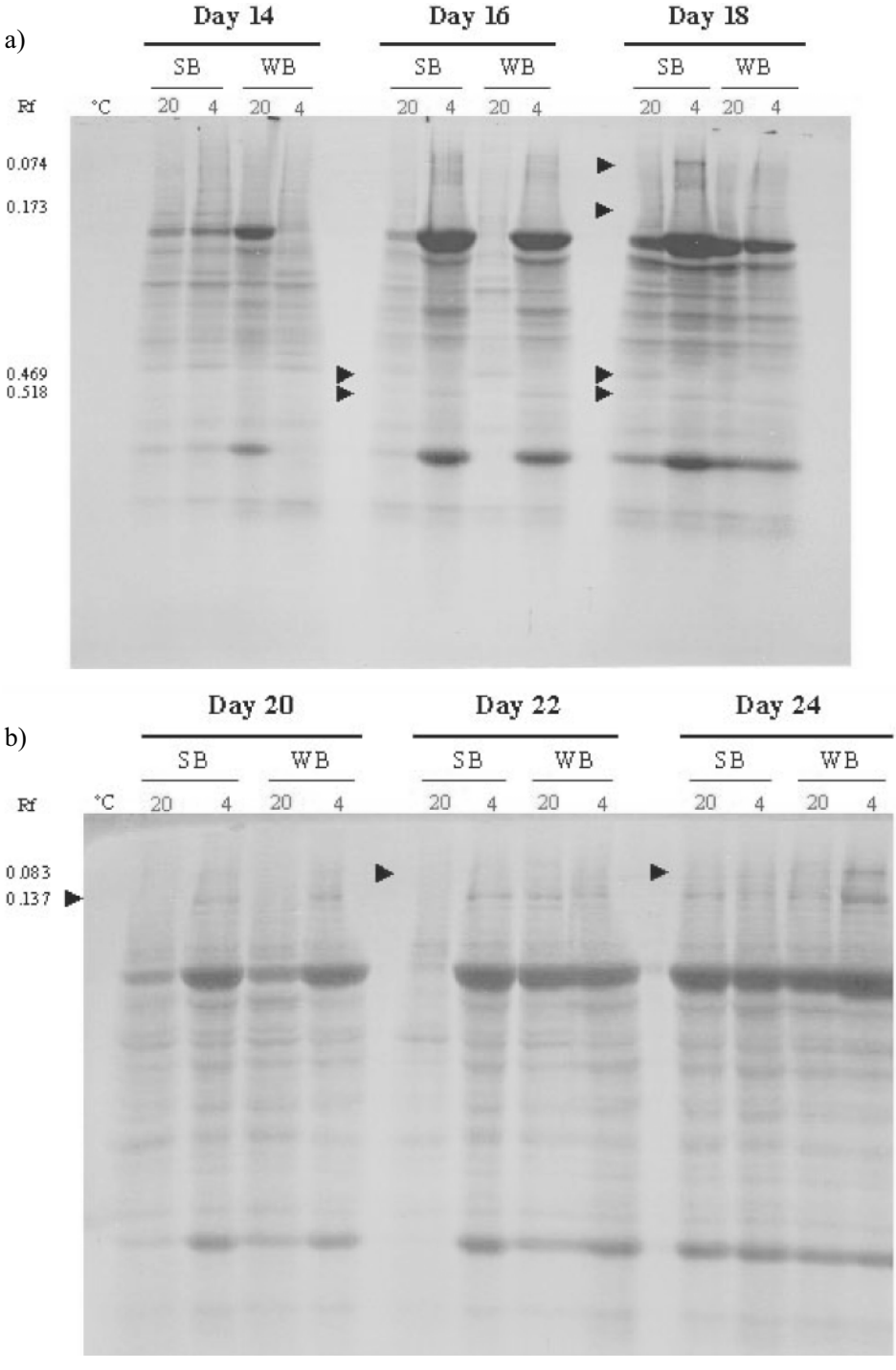
Fig. 2. The curve showing the changes of mean quantitative amount (mg/g Fw) of total leaf soluble proteins of a) spring barley cv. Reyhan and b) winter barley cv. Makouei grown either at a constant 20 °C (solid line with filled symbols) or at 20 °C followed by transfer to 4 °C on day 14 for 14 d and then transferred back to 20 °C on day 28 (dotted line with open symbols). The arrows show the timing of the dowshift and upshift of temperatures. Bars represent ±S.E. but where absent, the variation about the mean was less than the diameter of the symbol (n = 3). SB = Spring barley; WB = Winter barley; d = day

($P < 0.01$). The transfer of seedlings from 20 to 4 °C on day 14 resulted in significant increases in leaf total protein amounts regardless of cultivar during the second week of exposure to low temperature treatment compared to seedlings maintained throughout at a constant 20 °C (Fig. 2) but, such response was not recognized over the first week in either cultivar. On the other hand, the cold-induced increases in proteins were detected 2 d earlier in the cold-treated leaves of winter barley cultivar (experimental days 22–28 = 8–14 d at 4 °C) than in those of spring barley cultivar (days 24–28 = 10–14 d at 4 °C). The most protein's accumulation was detected on experimental day 24 (10 d at 4 °C) in the cold-treated leaves of the winter (27% increase) and of the spring (21% increase) barley cultivars compared with the controls. The return of cold treated seedlings to 20 °C resulted in the production of proteins in leaves to levels similar to those in seedlings maintained throughout at a constant 20 °C in both cultivars (days 30–40).

Changes in polypeptides detected on both cultivars at 4 °C

To check if there were any qualitative changes in polypeptide composition between cultivars, the total leaf soluble proteins were size-fractionated on SDS-PAGE. At the time of transfer of young seedlings from 20 to 4 °C, no obvious between-cultivar changes in soluble proteins were detected in the leaves of spring and winter barley cultivars. On days 16 and 18 (2 and 4 d at 4 °C), one polypeptide pattern ($rf = 0.469$) disappeared in the cold-treated leaves of both cultivars compared with the controls (Fig. 3a). On day 16, on the contrary, the pattern of $rf = 0.518$ was detected only in the 4 °C-treated leaves of both cultivars but not in the controls. At a lower density, such response was also distinguished on day 18 (4 d at 4 °C) in both cultivars. Moreover, at the latter sample time, one polypeptide ($rf = 0.074$) came into view in the leaves of cold-treated seedlings of both barley cultivars compared to the controls: this response was more obvious in the spring cultivar (Fig. 3a). Over days 16 and 18 (2 and 4 d at 4 °C), increases in the density of the $rf = 0.173$ protein pattern were detected in both cultivars compared with the controls.

On day 20 (6 d at 4 °C), an increase in the density of $rf = 0.137$ protein pattern (low density) was detected in seedlings transferred to the cold treatment in both cultivars compared to those in the control treatment (20 °C; Fig. 3b). Following 8 d at 4 °C (day 22), a new polypeptide ($rf = 0.083$) was recognized in the leaves of cold treated seedlings of winter barley (at a low density), but it was identified neither in their controls, nor in those of spring barley in both temperature treatments at this sample time (Fig. 3b). On day 24 (10 d following cold treatment), this new polypeptide ($rf = 0.083$) was observed in the leaves of both cultivars compared to the controls: its density was more recognizable in the winter barley cultivar (Fig. 3b). In other words, in the cold treatment, the winter cultivar responded 2 d earlier than the spring cultivar in the appearance of the new $rf = 0.083$ polypeptide. This polypeptide was observed in the leaves of the spring and winter barley until the end of cold period. In general, over the two-week cold exposure period (days 14–28), the most


a)
Day 14
Day 16
Day 18
SB
WB
Rf
°C
20
4
0.074
0.173
0.469
0.518
b)
Day 20
Day 22
Day 24
0.083
0.137

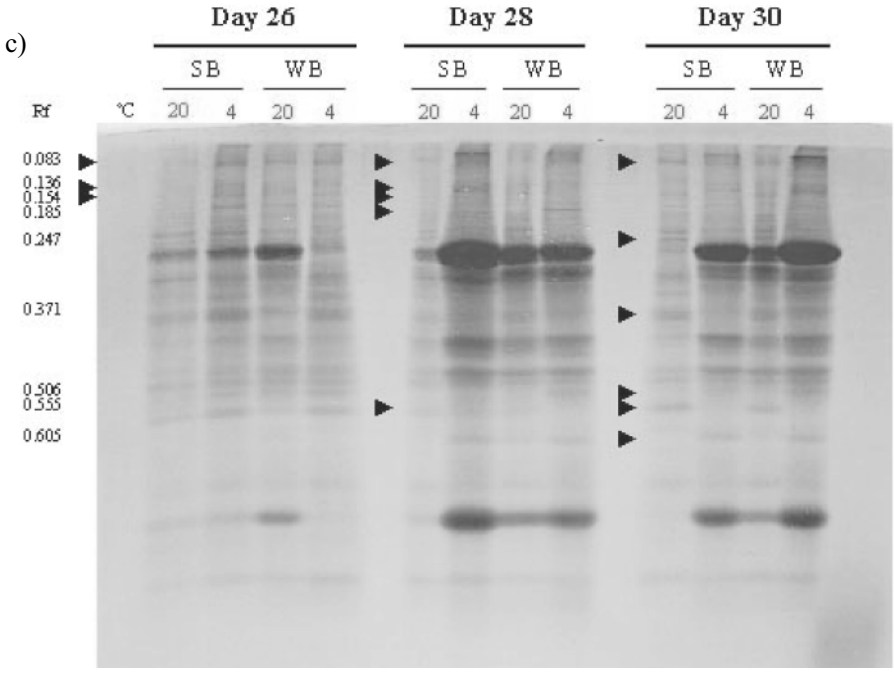


Fig. 3. SDS-PAGE patterns of total leaf soluble protein fractions from a spring barley (SB; *Hordeum vulgare* L.) cv. Reyhan and a winter barley (WB) cv. Makouei grown either at a constant 20 °C (shown as 20 °C), or at 20 °C followed by transfer to 4 °C on day 14 for 14 d and then transferred back to 20 °C on day 28 (shown as 4 °C). The arrows indicate bands which were induced on: a) the experimental days 14–18; b) experimental days 20–24; c) experimental days 26–30

appreciable changes in the leaf soluble protein patterns were occurred during the first week irrespective of cultivar. After the return of cold-treated seedling from 4 to 20 °C, the cold-treated leaves of both cultivars produced almost the same protein patterns as those of the controls (the patterns on days 32–40 are not shown).

DISCUSSION

Low temperature treatment effect on total leaf proteins

The two-week exposure of the 14-d old seedlings to a vernalization low temperature treatment (4 °C), resulted in considerable increases in total soluble proteins in the leaves of the spring and the winter barley cultivars (Fig. 2). Therefore, following the return of cold-treated seedlings to 20 °C, the total leaf proteins amount was similar to that in the controls maintained at 20 °C over the experimental period. Indeed, following the transfer of cold treated seedlings to 20 °C (i.e. days 30–40), protein accu-

mulation tailed off. Hence, we observed a clear between-temperature treatment difference within a cultivar during the cold treatment period. While both cultivars responded similarly to temperature alteration over the experimental period (i.e. days 14–40), the most considerable changes in proteins amount occurred during the second week of exposure to 4 °C: the winter barley cultivar responded 48 h earlier than the spring barley cultivar to temperature changes. This rapid response to a low temperature by the winter barley cultivar indicates a more sensitive response compared with the spring barley. In agreement with these data, Karimzadeh et al. [25] also reported a significant cellular protein accumulation during the first week (in particular 7 d at 4 °C) of low temperature treatment in winter wheat compared with spring wheat. In another research (unpublished data) on a spring and a winter Iranian wheat cultivars, using the same experimental protocol as reported here, undertaken in our department, clear cold treatment increases in total leaf proteins quantity occurred over the first week of exposure to a low temperature treatment (4 °C) irrespective of cultivar: the winter wheat cultivar responded more obviously and rapidly (over the first 48 h of low temperature) than the spring wheat cultivar to temperature changes. In the two latter references on wheat, the proteins accumulation occurred during the first week at 4 °C, indicating that spring and winter wheat cultivars (in particular winter cultivar) responded a week earlier than barley cultivar which responded over the second week of exposure period. This accumulation of proteins is in agreement with quantitative data on barley in this report, probably denoting the concentrated amount of proteins in the cells and tissues [25, 44]. Quantitative and qualitative differences in soluble protein amount between non-acclimated and cold-acclimated plants which were clearly observed in the present work, have also been found in cereal and non-cereal plants. For example, in recent another research carried out in our department, using spring and winter cultivars (Hyola 401 and Symbol, respectively) of canola seedlings exposed to 4 °C for 3 weeks, Karimzadeh et al. [24] reported clear cold-induced increases in protein amounts at low temperature treatment in winter canola but not in spring canola. These increases appeared 8 d following the cold exposure to 4 °C and were maintained until 2 d following the return to 15/10 °C (day/night) in the leaves of the winter canola cultivar.

Electrophoretic changes in total leaf proteins by low temperature

Changes in the electrophoretic patterns of the total leaf soluble proteins showed between-temperature treatments differences within a cultivar and between-cultivar differences. Over the cold exposure period, some of the polypeptide patterns (e.g. rf = 0.469) were only observed in the control seedlings maintained throughout at 20 °C, but not in the cold-treated seedlings of both cultivars. This may indicate that this polypeptide was initially sensitive to cold treatment and, hence, disappeared at the low temperature treatment over the first days of transfer to cold treatment (2 and 4 d at 4 °C). On the other hand, there was other polypeptide (rf = 0.518) which was produced at the early days (2 d at 4 °C) only in the cold treatment seedling leaves of

both cultivars, but not in the controls. A new rf = 0.074-polypeptide was only detected at the cold treatment in both cultivars. This polypeptide perhaps correlates positively with cold hardiness in barley cultivars, in particular in winter cultivar which responded earlier to cold stress [10].

In another experiment undertaken in our department on wheat, between-cultivars and between-temperature treatments, differences in electrophoretic protein patterns were also identified. In wheat materials ten new cold-induced polypeptides ranging from 17 to 200 kDa were produced over the first week at 4 °C regardless of spring and winter wheat cultivars (unpublished data). In the present study on barley, only a new cold-induced rf = 0.074, polypeptide was detected at the 4 °C-treatment in both cultivars. This cold-induced response may be correlated with cold acclimation. Limin et al. [30] also reported that in wheat and other cereals, the expression of several genes during cold acclimation was found to be positively correlated with the capacity of each genotype to develop freezing tolerance.

On the other hand, researchers pointed out that the disappearance of some protein bands and the expression of other proteins are part of metabolic regulations that plants show in response to cold stress and may not be correlated with cold hardiness of plant [14, 41]. The accumulation of proteins and both the production of new cold-induced proteins and the changes in the density of some polypeptides in response to low temperature treatment have been reported by numerous researcher in wheat, rye, barley, alfalfa and canola (see Introduction). The accumulation of an 80-kDa DHN-like protein (P-80) was reported by Bravo et al. [4] in barley under cold acclimation 6/4 °C (day/night) in cold-acclimated leaves. Furthermore, the 14 kDa COR14-cold-regulated protein is a polypeptide which accumulated under low temperature (4 °C) conditions in the chloroplasts of barley leaves [11]. Its related gene, cor14b [9], is expressed in barley's and in the related cereals' leaves, during plant exposure to low temperatures. Another low temperature-regulated protein in barley is COR75, the predominant COR protein. This protein of 75 kDa which has been investigated in many barley genotypes [34, 37] is accumulated faster during the first-leaf stages at low temperatures in frost resistant varieties when compared with sensitive varieties. In general, the expression of COR75 in response to various environmental stresses was recognized in plant roots and leaves at the first-leaf stage [10].

In conclusion, our results show that soluble proteins accumulation occurred at low temperature during the second week of exposure to 4 °C irrespective of barley cultivar: the winter cultivar was 2 d prior in this response. The electrophoretic studies showed that a new cold-induced polypeptide was recognized in the leaves of winter barley cultivar on day 22 (8 d at 4 °C) compared to the control: this polypeptide appeared 2 d earlier in the winter cultivar compared with the spring cultivar at 4 °C. Actually, we are studying to determine cold-shock proteins are part of this protein accumulation in our cereal-related cultivars. This should provide further clues about the mechanisms that result in gaining informations about Iranian barley and wheat cultivars with different growth habits.

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