Journal of Plant Physiology **165** (2008) 1142—1151

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JOURNAL OF PLANT PHYSIOLOGY

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Expression of dehydrin 5 during the development of frost tolerance in barley (*Hordeum vulgare*)

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Received 24 August 2007; received in revised form 3 October 2007; accepted 5 October 2007

KEYWORDS
Cold acclimation;
Dehydrin 5;
Frost tolerance;
Growth habits;
Hordeum vulgare

Summary

The *Dhn5* gene is the major cold-inducible dehydrin gene in barley. This study deals with the relationship between *Dhn5* gene expression and its protein product accumulation, and the development of frost tolerance (FT) upon cold acclimation (CA) in 10 barley cultivars of different growth habits and geographical origins. The activation of *Dhn5* gene expression was determined by quantitative reverse transcription-polymerase chain reaction (qRT-PCR), the accumulation of DHN5 protein was evaluated by protein gel blot analysis using a specific anti-dehydrin antibody, and the acquired level of FT was determined by a direct frost test. During the first 2 weeks of CA, there was a rapid increase in *Dhn5* gene expression, DHN5 protein accumulation and FT in all cultivars examined. After 2 weeks of CA, differences in DHN5 accumulation and in FT measured as lethal temperature (LT_{50}) were observed between the cultivars belonging to different growth habits. Specifically, intermediate (I) and winter (W) cultivars showed a higher level of DHN5 accumulation and FT than the spring (S) cultivars, which exhibited a lower level of accumulated DHN5 and FT. (Intermediate cultivars do not have vernalization requirement, but they are able to induce a relatively high level of FT upon CA.)

Abbreviations: CA, cold acclimation (cultivation at 3 °C); *Cor*, cold-regulated genes; *Dhn*, dehydrin gene; FT, frost tolerance; FW, fresh weight; I, intermediate cultivar; *Lea*, late embryogenesis abundant genes; LT_{50}, lethal temperature when 50% of the sample dies; qRT PCR, quantitative reverse transcription-polymerase chain reaction; RE, relative expression (of *Dhn5* mRNA); S, spring cultivar; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; *Vrn1*, vernalization gene 1; W, winter cultivar; *Wcs120*, wheat cold-specific 120 gene.

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0176-1617/$ - see front matter
doi:10.1016/j.jplph.2007.10.009

In contrast, no differences between the cultivars belonging to different growth habits in *Dhn5* mRNA accumulation were found. After 3 weeks of CA, the differences in accumulated DHN5 and FT between the individual growth habits became evident due to different developmental regulation of FT. The amount of accumulated DHN5 corresponded well with the level of FT of individual cultivars. We conclude that the amount of accumulated DHN5 after a certain period of CA differed according to the growth habits of cultivars and can be used as a marker for determination of FT in barley.

Introduction

Dehydrins (LEA II family) are either constitutively present or stress-inducible proteins widely spread in autotrophic organisms ranging from cyanobacteria to angiosperms. They accumulate in various plant tissues upon stress conditions with a dehydrative component, i.e., drought, salinity, enhanced evaporation, heat, frost and cold (Close, 1996, 1997; Svensson et al., 2002; Allagulova et al., 2003; Rorat, 2006). The functions of dehydrins in plant cells in reaction to stress conditions have not yet been precisely elucidated; however, some experiments to uncover their roles have been conducted. The results of these findings have recently been reviewed in Rorat (2006), and in Kosová et al. (2007) for cold stress.

The expression of some dehydrins is specifically induced by cold. However, large differences in the expression level of a given dehydrin gene can be observed in cultivars of the same species differing in frost tolerance (FT). Differences in the expression of various *Lea* genes in wheat and barley cultivars, which differ in FT, have been observed by many authors (e.g., Zhu et al., 2000; Kobayashi et al., 2004).

In barley, 13 dehydrin genes, *Dhn1* to *Dhn13*, have been described to date (Rodriguez et al., 2005). Under cold, the induction of *Dhn5* and *Dhn8* genes has been detected at the transcription level (Choi et al., 1999; Zhu et al., 2000). At the protein level, a significant accumulation of DHN5 protein has been observed on protein gel blots (Van Zee et al., 1995; Bravo et al., 1999, 2003; Zhu et al., 2000) using a specific anti-dehydrin antibody (Close et al., 1993). It has been long known that the *Dhn5* gene is an orthologue to the *Wcs120* gene in wheat, a member of the *Wcs120* gene family (for review on *Wcs120* gene family, see Sarhan et al., 1997). The WCS120 protein in wheat is exclusively cold inducible, and its level of accumulation corresponds well with the acquired level of FT in the wheat tissue. Thus, the WCS120 protein is considered a marker of FT in wheat (Houde et al., 1992). Recently, Vítámvás et al. (2007) distinguished two differentially frost-tolerant winter wheat cultivars – Mironovskaya 808 and Bezostaya – on the basis of different amounts of accumulated WCS120 proteins after 3 weeks of cold acclimation (CA).

Because the DHN5 protein is structurally very similar to the WCS120 protein in wheat, its crucial function in the acquisition and development of FT in barley can be assumed. Many authors have dealt with this problem, but to date, no clear model of the role of DHN5 in the dynamics of FT in barley has been proposed. Van Zee et al. (1995) found no differences in the accumulation of DHN5 between the winter cultivar Dicktoo and the spring cultivar Morex after 1, 2, 4, 8 and 24 d of CA (2 °C), while Zhu et al. (2000) found differences in FT measured as LT_{50} (lethal temperature when 50% of the sample dies) values, and in the accumulation of DHN5 between the same cultivars over 14 d at 4 °C. Bravo et al. (1999) compared the accumulation of DHN5 after 6 and 30 d of CA in three barley cultivars with their FT in a non-acclimated state measured as LT_{50} values, and found that the cultivar with the lowest FT had the lowest accumulation of DHN5. However, the relationship between FT and the accumulation of DHN5 in the other two cultivars was reversed.

It has been long known that barley cultivars belonging to all growth habits can increase their FT upon CA. However, individual growth habits differ in their ability to maintain enhanced FT across time. Winter cultivars, which have a vernalization requirement, can retain a relatively high level of FT until the fulfillment of vernalization requirement, i.e., until the *vrn1* gene is expressed. In contrast, spring cultivars begin to express Vrn1 gene products early in their individual development, and can thus induce an enhanced level of FT only transiently. Intermediate cultivars also do not have vernalization, but they are able to induce a relatively high level of FT upon CA, especially under neutral or short-day photoperiods since these light regimes repress the expression of *vrn1*. It has been confirmed by many researchers (e.g., Danyluk

et al., 2003; Kane et al., 2005; Kobayashi et al., 2005) that the Vrn1 gene product 'switches' the transition of the *Triticae* into the less stress-tolerant reproductive phase of their individual development, which is accompanied by a decrease in the expression of many *Cor* genes, including dehydrins.

In summary, to date, cold-specific *Dhn5* gene expression and its corresponding protein product accumulation have been confirmed in barley. Distinctions in DHN5 accumulation between spring and winter cultivars have been detected during the initial phase of CA by some authors. Others, however, have obtained contradictory results under similar growth conditions. On the other hand, the expression of *Dhn5* mRNA in cultivars of different FT has not yet been quantitatively evaluated. Moreover, no quantitative relationship between DHN5 accumulation and FT has been described. Thus, the aim of our study was to elucidate the dynamics of *Dhn5* mRNA and DHN5 protein accumulation and FT development in the three barley growth habits during the first 2 weeks of CA, as well as to define the relationship between the level of DHN5 accumulation and the acquired level of FT more precisely.

Material and methods

Experimental design

For our purposes, 21 barley cultivars of different geographical origin representing all main growth habits (intermediate – I, winter – W, spring – S) were chosen. For the names of the cultivars, the abbreviations used here (in Figure 3) and their geographical and physiological characteristics, see Table 1.

The seeds were put on moist filter paper and then allowed to germinate for 2 d at 21 °C in the dark. Fully germinated seeds were planted into pots (15 seeds per pot) of 10 × 10 cm and cultivated at 17 °C and 12 h photoperiod (400 μmol m^{-2} s^{-1}) in growth chamber (Tyler, type T-16/4, Budapest, Hungary) until the three-leaf stage. The temperature was then decreased to 3 °C. Young leaves were collected for mRNA and protein analysis at 0, 0.5, 1, 3, 7, 14 and 21 d of CA. At the same time, some plants were collected for FT tests.

Determination of frost tolerance

Plants of the individual cultivars were divided into five groups consisting of eight to ten plants and exposed to −4 °C for 20 h, followed by five different freezing temperatures in separate freezers for 24 h. The temperatures differed by 2 °C and the rate of cooling and

Table 1. Twenty-one barley cultivars, their geographical origin, growth habit, number of rows in an ear and their frost tolerance determined as LT_{50} values after a 3-week CA

Cultivar	Abbreviation	Origin	Growth habit	Ear	LT_{50} (°C)
Lunet	Ln	CZE	I	Six-row	−15.6a
Dicktoo	Dc	USA	I	Six-row	−15.3ab
Luxor	Lx	CZE	W	Six-row	−15.2ab
Okal	Ok	CZE	W	Six-row	−15.1ab
Hutorok	Hu	RUS	W	Six-row	−14.8abc
Tiffany	Ti	DEU	W	Two-row	−14.5bc
Luran	Lr	CZE	W	Six-row	−14.4c
Kromir	Ko	CZE	I	Six-row	−14.3cd
Kromoz	Km	CZE	W	Six-row	−14.3cd
Campill	Ca	DEU	W	Six-row	−14.2cd
Vilna	Vi	NLD	W	Two-row	−13.8de
Jolante	Jl	DEU	W	Two-row	−13.5de
Igri	Ig	DEU	W	Two-row	−13.4de
Duet	Du	GBR	W	Two-row	−13.1e
Atlas68	At	USA	S	Six-row	−11.5f
Prestige	Pr	FRA	S	Two-row	−11.2f
Jotun	Jt	NOR	S	Six-row	−11.0f
Braemar	Br	GBR	S	Two-row	−10.9fg
Diamant	Da	CZE	S	Two-row	−10.9fg
Sebastian	Se	DNK	S	Two-row	−10.9fg
Amulet	Am	CZE	S	Two-row	−10.0g

Statistically significant differences ($LSD_{0.05}$) in LT_{50} values between individual cultivars are marked with different letters. The cultivars are ordered according to their LT_{50} values (in descending order).

Abbreviations: CZE – the Czech Republic, DEU – Germany, DNK – Denmark, FRA – France, GBR – Great Britain, NLD – the Netherlands, NOR – Norway, RUS – Russia, USA – the United States of America.

thawing was 2 °C h^{-1}. After thawing, the plants were grown in soil in a greenhouse at a temperature of approximately 20 °C. After 3 weeks, the plant survival rates were determined (%) for the particular freezing temperatures. FT was expressed in LT_{50} values (lethal temperature of 50% of the sample), calculated according to the model of Janáček and Prášil (1991).

mRNA isolation and qRT-PCR

Total RNA was extracted from 50 mg of leaf tissue using Ambion RNAqueous™ Kit. DNA contaminations were cut by Turbo DNA free™ (Ambion). Single-stranded cDNA was prepared from 500 ng of total RNA using the QuantiTect® Reverse Transcription Kit (Qiagen). All reactions were performed according to the standard protocols. Gene quantification was performed using real-time PCR. Specific primer pairs of studied genes were designed based on sequences presented in the GenBank database (AF043096), using Primer 3 software. *Dhn5*: F 5′-AGC AGA CAG GTG GCA TCT AC-3′, R 5′-GCAGCTTGTCCTTGATCTTG-3′ (380 bp).

Each reaction was performed with 5 μL of 1:10 (v/v) dilution of the first-strand cDNA (corresponding to 25 ng isolated total RNA) in a total reaction volume of 25 μL using the SybrGreen PCR Kit (Qiagen) with the following final concentration of constituent components: 1 × QuantiTect SYBR Green PCR Master Mix, primers, each of them, 0.25 μM, uracil-*N*-glycosylase 0.25 U. Reaction conditions for thermal cycling were the following: starting with a denaturation step of 95 °C for 15 min, followed by 32 cycles of 94 °C for 15 s, 58 °C for 30 s and 72 °C for 30 s. Amplification specificity was checked with a heat-dissociated protocol (melting curves in 58–90 °C range), as a final step of the PCR. Fragment of barley α-tubulin was used as an internal control for the relative amount of RNA. Barley gene α-tubulin amplified with the specific primers F 5′-AGTGTCCTGTCCACCCACTC-3′ and R 5′-CCAAGGATCCACTTGATGCT-3′ (acc. no. U40042) was used as a constitutive control. The amplification of this gene was done under the reaction conditions published in Suprunova et al. (2004).

Transcription activity was evaluated as normalized relative expression calculated with qPCR efficiency correlation in accordance with the method of Pfaffl (2001). The sample with the highest expression level was considered as an internal calibrator. Efficiency of all reactions was calculated from the direction of the calibration curve. For each sample, changes in the activity of the *Dhn5* gene were calculated in relation to the expression of this gene under optimal growth conditions (21 °C). Each sample was collected from three to five plants and examined in triplicate; each value is the mean ± standard error (SE).

Protein analysis

The young leaves were frozen in liquid nitrogen and stored at −80 °C. The tissue was homogenized with extraction buffer (100 mM Tris–HCl, pH 9 containing "Complete EDTA-free Protease Inhibitor Cocktail Tablets" (Roche, Basel, Switzerland)) under liquid nitrogen using the mortar and pestle. The amount of the extraction buffer was dependent on the fresh weight (FW) of the sample; e.g., 5 mL of the buffer was added to 1 g FW of the sample. The mixture was centrifuged twice at 20,000*g* at 4 °C for 20 min, then kept in boiling water for 15 min, cooled rapidly to 4 °C and centrifuged at 20,000*g* (4 °C) for 20 min. Concentration of heat-stable proteins was determined according to Bradford (1976). The supernatants were then precipitated by cold acetone with 1% 2-mercaptoethanol (v/v) in 1:5 sample:acetone ratio (v/v). The pellet was then centrifuged at 20,000*g* (4 °C) for 20 min and dried.

Dry samples were resolved in SDS-sample buffer prepared according to the manual of Biometra (Göttingen, Germany) – to 200 μL of the original sample, 750 μL of the sample buffer was added. The samples were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10% resolving gel) (Laemmli, 1970), 5 μL of the sample per line. SDS-PAGE was carried out on apparatus of Biometra. The SDS-PAGE was run at 10 mA per gel (stacking gel) and at 25 mA per gel (resolving gel). For the estimation of the DHN5 band, SDS-PAGE Colored Standards, broad range (Bio-Rad, Hercules, CA, USA), were used.

Protein gel blots were carried out on a semi-dry blotter (Biometra) for 1.5 h at 1 mA cm^{-2} using a nitrocellulose membrane (Bio-Rad). Membranes were blocked in 3% gelatin in Tris-buffered saline (TBS) (w/v; 20 mM Tris–HCl, pH 7.5; 500 mM NaCl) for 2 h, washed in TTBS (0.1% (v/v) Tween-20 in TBS) for 10 min, and incubated in anti-dehydrin antibody dissolved in 1% gelatin in TTBS (w/v; dilution 1:1000) overnight. After the wash in TTBS for 10 min, the membranes were incubated in goat-anti-rabbit-alkaline phosphatase secondary antibody in 1% gelatin in TTBS (w/v; dilution 1:3000) for 2 h. After final washes in TTBS and TBS (each for 10 min), the membranes were developed in the AP Conjugate Substrate Kit (Bio-Rad) until the reaction was completed (ca. 5–7 min).

Densitometric analyses of the amount of accumulated DHN5 on gel blots were performed using the program ElfoMan 2.6 (Semeckỳ).

Statistical analysis

Statistical evaluation was carried out on the basis of a multiple range test (LSD at the 5% significance level) of averages calculated from four repetitions from two different samples (extractions) (*Unistat version 5.1.*, *Unistat Ltd.*, London, UK). Variability between individual repetitions was expressed by SE.

Results

Dynamics of *Dhn5* gene expression, DHN5 protein accumulation and FT development during the first 14 d of CA

In this section, we present the results obtained on ten cultivars representing different growth

habits – I (cvs. Dicktoo, Kromir, Lunet), W (cvs. Igri, Okal, Tiffany, Vilna) and S (cvs. Amulet, Atlas 68, Braemar), which exhibit different levels of FT.

The rate of relative *Dhn5* mRNA expression in the three growth habits during the first 14 d of CA is shown in Figure 1A. Initiation of *Dhn5* gene expression was evident 12 h after the beginning of CA. The level of *Dhn5* expression continually increased during the first d of CA, and the maximum level of *Dhn5* expression was reached 4–5 d after the beginning of CA. After that, it decreased, but did not cease totally until the 2-week period. No statistically significant differences between the cultivars belonging to different growth habits were found over the entire experiment. However, some slight, but statistically non-significant differences were observed. The highest relative expression level during the experiment was detected in spring cultivars on the 7th day of CA. However, differences between individual cultivars occurred. In the samples taken after 12 h of cold, the highest relative activity of *Dhn5* expression was detected in the most frost-tolerant cultivars Dicktoo and Lunet. The frost-tolerant cultivars also reached the maximum *Dhn5* relative expression earlier (about 3rd day of CA) than the frost-susceptible ones (about the 7th day of CA). Some winter cultivars, however, showed a relatively low level of *Dhn5* expression compared with some spring cultivars during the experiment. For example, the frost-tolerant winter cultivar Okal exhibited a lower level of *Dhn5* relative expression than the frost-susceptible spring cultivars Atlas 68 and Amulet.

The amounts of accumulated DHN5 protein in the three growth habits during the first 2 weeks of CA are given in Figure 1B, and the amounts of DHN5 in the individual cultivars are shown in Figure 2. The accumulated DHN5 protein was already detectable in all cultivars after 1 day of CA. After 1 and 3 d of CA, the amount of accumulated protein was very similar in all cultivars examined. After 1 and 2 weeks of CA, differences in the amount of accumulated DHN5 protein between the cultivars

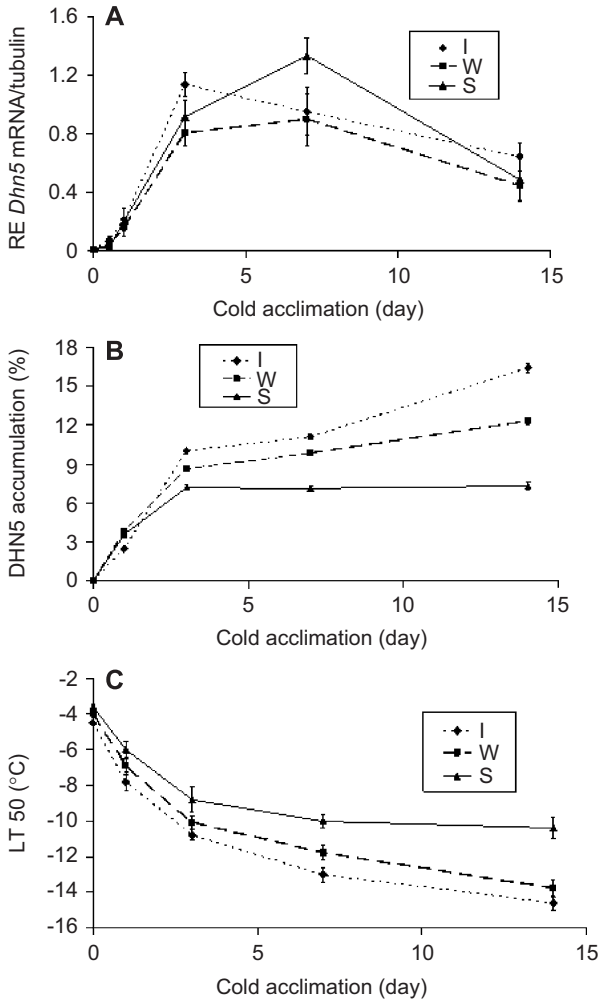

Figure 1. The kinetics of relative expression (RE) of *Dhn5* mRNA accumulation (A), DHN5 protein accumulation (B) and the dynamics of FT development (C) during the first 14 d of CA in 10 cultivars representing the three growth habits. The values of *I* are means from the values obtained on cultivars Dicktoo, Kromir and Lunet, the values of *W* are means from the values obtained on cultivars Igri, Okal, Tiffany and Vilna, and the values of *S* are means from the values obtained on cultivars Amulet, Atlas 68 and Braemar. Vertical bars indicate SE ($n = 4$). In (B), the total of all values is 100%.

Figure 2. The dynamics of DHN5 accumulation in 10 barley cultivars belonging to different growth habits after 0 (control plants), 1, 3, 7 and 14 d of CA. The cultivars are ordered according to their LT_{50} values (in descending order).

belonging to the three growth habits (I, W and S) became detectable, with intermediate and winter cultivars having accumulated significantly higher amounts of DHN5 than the spring ones. However, a relatively high variability between individual cultivars was found (e.g., the spring cultivar Atlas 68 accumulated nearly the same amount of DHN5 as the winter cultivars even after 14 d of cold).

The dynamics of FT (expressed as LT_{50} values) development expressed as the mean values for the individual growth habits during the first 2 weeks of cold treatment are given in Figure 1C. During the first week of CA, the LT_{50} values of all cultivars examined decreased steeply and in a similar manner in all the cultivars of different growth habits. After 2 weeks, the rate of decrease in LT_{50} values was slower, and differences between individual growth habits began to occur. The LT_{50} values of intermediate cultivars decreased more rapidly than the LT_{50} values of the winter cultivars, while the LT_{50} values of the spring cultivars decreased only slightly.

Relationship between DHN5 accumulation and FT level after 21 d of cold treatment

The level of DHN5 accumulation in 21 barley cultivars after 21 d of CA is given in Figure 3, and its relationship to the level of FT of the individual cultivars is shown in Figure 4. The maximum level of FT was reached after 3 weeks of CA (see Table 1). At the protein level, significant differences in DHN5 protein accumulation between the cultivars belonging to different growth habits occurred, with intermediate and winter cultivars having accumulated significantly higher amounts of DHN5 than the spring ones. Moreover, a significant linear correlation between the amount of DHN5 protein and the

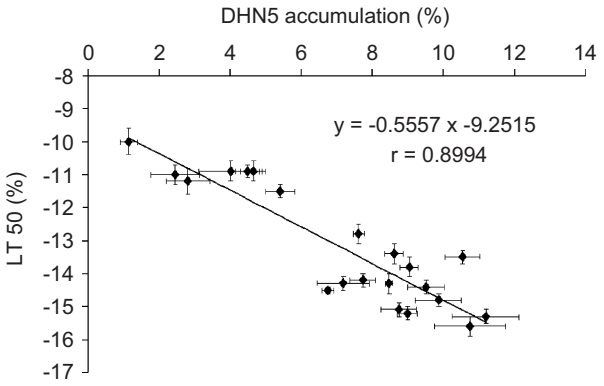

Figure 4. The relationship between DHN5 accumulation (mean values) and FT in 21 barley cultivars after 21 d of CA. Both horizontal bars and vertical bars indicate SE ($n = 4$). The level of DHN5 accumulation is expressed relatively in percent; 100% = total amount of DHN5 in 21 cultivars. Variability in LT_{50} values of the individual cultivars is also given in Table 1.

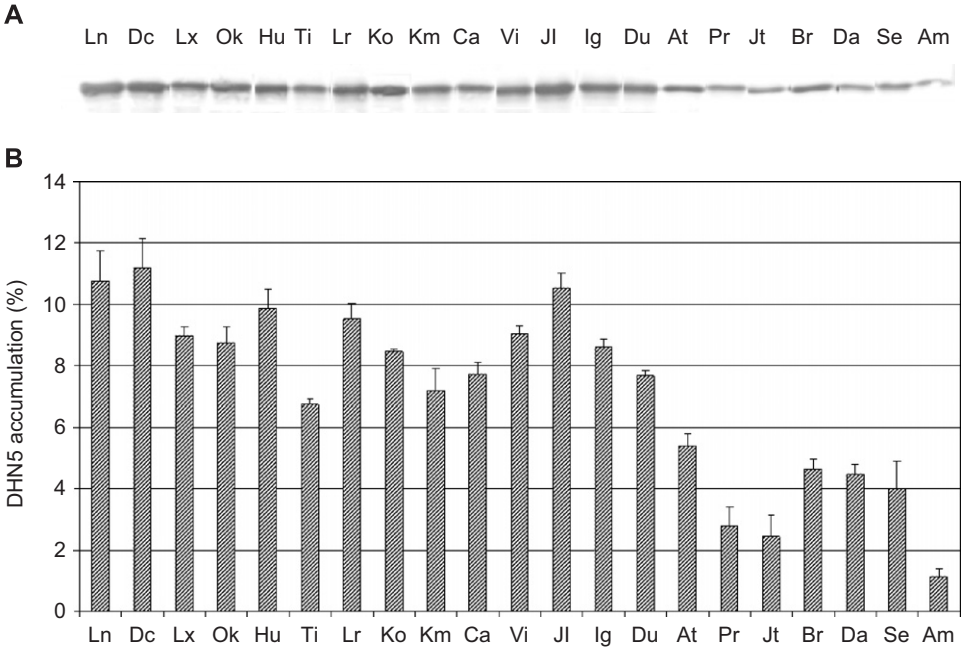

Figure 3. The accumulation of DHN5 in 21 cultivars after 21 d of CA. Protein gel blot (A) and its densitometric analysis (B). The cultivars are ordered according to their LT_{50} values after 21 d of CA (in descending order). The columns represent means obtained from four repetitions, the vertical bars represent SE ($n = 4$). The level of DHN5 accumulation is expressed relatively in percent; 100% = total amount of DHN5 in 21 cultivars.

acquired level of FT (expressed as LT_{50}) was found; the correlation coefficient was $r = 0.9$.

Discussion

During cold treatment, significant physiological and biochemical changes were observed in our experiments. We were able to detect *Dhn5* mRNA after 12 h of CA. This result is in accordance with those observed by other authors who reported an early induction of cold-responsive genes after the application of CA. The expression of *Dhn5* gene in barley after a few hours of CA has been described by Zhu et al. (2000). Some authors (Choi et al., 1999) have also detected quantitative differences in the level of *Dhn5* gene expression between differently frost-tolerant cultivars. However, the results of our experiments did not confirm expected differences (Choi et al., 1999; Suprunova et al., 2004) in the regulation of *Dhn5* gene expression between stress-tolerant and stress-susceptible cultivars. In contrast, some frost-susceptible spring cultivars (Amulet, Atlas 68) showed higher levels of *Dhn5* expression than some relatively frost-tolerant winter cultivars (Okal), although these differences were not statistically significant. This discrepancy, i.e., high levels of *Dhn5* expression in frost-susceptible cultivars compared with the frost-tolerant ones, can be explained by the existence of an alternative transcriptional regulatory network of the genes involved in low temperature and dehydration response (for this topic, see Yang et al., 2005).

The accumulation of DHN5 protein increased very rapidly during the first 2 weeks of cold. On the 1st and 3rd d of CA, no differences between the growth habits were observed. However, the differences in DHN5 accumulation between highly frost-tolerant intermediate and winter cultivars and less frost-tolerant spring cultivars became evident after 1 week of CA, and became more pronounced after 2 weeks of CA. These results modify the conclusions previously published by Van Zee et al. (1995), who compared the accumulation of DHN5 (86 kDa protein) in the winter cultivar Dicktoo and the spring cultivar Morex after 1, 2, 4, 8 and 24 d of CA (2 °C) and found no differences in DHN5 accumulation over the entire course of the experiment. In contrast, Zhu et al. (2000) found difference in DHN5 accumulation between the winter cultivar Dicktoo and the spring cultivar Morex in all samples (the first sampling was conducted on 0.5 d of CA) during the whole first 2 weeks of CA. Our results indicate that the frost-tolerant intermediate and winter barley cultivars, as well as the frost-susceptible spring ones, start accumulating DHN5 protein at the same time after the beginning of CA. However, they begin to differentiate in the amount of accumulated DHN5 throughout CA when intermediate and winter cultivars begin to accumulate larger amounts of DHN5 than the spring ones. The obtained results, i.e., the same level of accumulated DHN5 in differently frost-tolerant barley cultivars at the beginning of CA, correspond with our previous results (Vítámvás et al., unpublished results) where a similar induction of DHN5 accumulation in spring cultivar Atlas 68 and a highly frost-tolerant winter cultivar Luxor was observed having used a set of different induction temperatures (17, 9 and 4 °C) – the accumulated DHN5 became detectable at the same temperature (17 °C) in both cultivars, while in wheat the highly frost-tolerant cultivar Mironovskaya 808 began to accumulate WCS120 protein at higher induction temperature (17 °C) than the less-tolerant winter cultivars Šárka, Zdar and Bill, and analogously, the frost-tolerant winter cultivars began to accumulate WCS120 at higher induction temperature (9 °C) than the frost-sensitive spring cultivar Sandra.

When the mechanisms of change in protein synthesis are considered, it should be taken into account that CA leads to profound changes not only in the metabolism of many structural proteins but also in the metabolism of various components of protein synthesis machinery. Baldi et al. (2001) have detected increased levels of mRNA of one elongation factor (EF1Bβ) and two ribosomal proteins (RPS7 and RPL7A) in wheat and barley following exposure to 3 °C.

The differences in *Dhn5* gene expression and DHN5 protein accumulation (i.e., no differences between individual barley cultivars belonging to different growth habits were observed on the mRNA level, while on the protein level the differences between the cultivars gradually emerged during the time) are quite interesting. Analogous results were obtained by Zhu et al. (2000), who detected significant differences between Dicktoo and Morex barley during a 14-day CA at 4 °C on the DHN5 protein level, but no differences between the same cultivars upon the same cold treatment on the *Dhn5* mRNA level. However, they used only RT-PCR, while we used the more precise quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and a larger number of cultivars for determination of the level of *Dhn5* mRNA. Therefore, our analysis provides more reliable evidence that there are no significant differences in the accumulation of *Dhn5* mRNA during the first 2 weeks of CA between cultivars belonging to

different growth habits. The same authors have even detected samples where the DHN5 protein was present, but *Dhn5* mRNA was absent during a series of samplings of the cultivars mentioned above throughout the winter (cultivars grown under field conditions). The authors explained this fact by different stabilities of DHN5 protein and its corresponding mRNA (i.e., the protein is more stable than the mRNA) and by different kinetics of protein and mRNA accumulation. One possible explanation of the discrepancy observed by us, i.e., no differences between the cultivars in *Dhn5* mRNA accumulation vs. significant differences between the same cultivars in DHN5 protein accumulation, can also be based on different stabilities of DHN5 protein in differently frost-tolerant cultivars. It can be assumed that DHN5 protein is perhaps more stable in the more frost-tolerant cultivars than in the less frost-tolerant ones. To confirm this hypothesis, further experiments focused on the kinetics of DHN5 degradation need to be conducted.

Another explanation may lie in the differences in post-transcriptional control mechanisms in different barley cultivars, which can cause nearly the same mRNA level results in different protein levels in the cultivars. The difference between the mRNA and the protein level has been previously observed by Kirch et al. (1997) in the dehydrin gene *ci7* in potato, whose expression on the transcription level is induced by cold. However, the corresponding protein does not accumulate under the same conditions. This observation was confirmed by the authors using the GUS reporter gene fused with the *ci7* promoter in transgenic tomato. Under cold, GUS activity was not detected in the tomato tissue despite the presence of GUS mRNA.

The observed increase in FT, accompanied by the accumulation of specific cold-inducible dehydrins, is in accordance with our previous findings in wheat (Vítámvás et al., unpublished results) and with findings of other authors dealing with this problem in wheat and barley. The marked decrease in LT_{50} values during the beginning of cold treatment has been described by many researchers in barley (Zhu et al., 2000; Fowler et al., 2001; Prášil et al., 2007) and wheat (Fowler et al., 1996). The differences between intermediate, winter and spring cultivars, which increased gradually with the duration of CA, were observed in our experiments when spring cultivars started inducing FT comparable to the winter and intermediate ones. However, they ceased increasing the FT with the progression of CA earlier than the winter and intermediate cultivars. Similar results have recently been obtained by Limin et al. (2007) on five barley cultivars of winter and spring growth habits under CA upon long-day and short-day photoperiods.

After 3 weeks of CA, the highly frost-tolerant intermediate and winter cultivars showed higher levels of DHN5 accumulation than the frost-sensitive spring ones. One possible explanation of this phenomenon lies in the fact that spring cultivars can induce an increased level of FT only transiently because they start expressing the Vrn1 gene product in the early stages of their individual development, while intermediate and winter ones can retain high levels of FT for a longer time because they can postpone the developmental transition into the less stress-tolerant reproductive phase to the later stages of their individual development. Similar results were achieved by Bravo et al. (1999), who observed different levels of DHN5 accumulation after 30 d of cold in three barley cultivars differing in FT. However, they did not find a clear correlation between the level of FT (expressed as LT_{50}) of the cultivars and the amount of DHN5 accumulation, perhaps because they worked only with a small set of cultivars and compared the amount of DHN5 after 30 d of CA with FT in a non-acclimated state. They found that the most frost-tolerant cultivar accumulated a lower amount of DHN5 than did the second most frost-tolerant one. We also found variation between the level of FT and the amount of DHN5 in the individual cultivars. However, our results on 21 differently frost-tolerant cultivars showed a clear correlation between the amount of accumulated DHN5 protein and cultivars' FT after 3 weeks of CA (the time when maximum FT is reached). We can thus conclude that a correlation (in the form of a linear regression) between these two features exists, and that specifically, the cultivars that accumulate higher amounts of DHN5 exhibit a higher level of FT. It becomes evident that the *Dhn5* gene can be used as a marker of FT in barley in a way analogous to its orthologue, the *Wcs120* gene, in common wheat. However, it should be emphasized that we obtained the correlation between the amount of accumulated DHN5 protein and the level of FT only when we used the data of the cultivars representing all growth habits (or at least winter vs. spring cultivars). When the data obtained only on winter cultivars or on the spring cultivars were used for the calculations, no significant correlation between the DHN5 amount and the LT_{50} values was observed. Therefore, a relatively large data set obtained on cultivars with contrasting levels of FT is necessary to obtain this correlation.

It can be concluded that we have confirmed a cold-specific expression of *Dhn5*, as no *Dhn5* mRNA or DHN5 proteins were present in barley tissues

under optimum growth temperatures, while the presence of *Dhn5* mRNA was clearly detectable after 12 h of CA, and the presence of DHN5 protein became detectable after 24 h of CA. No differences in *Dhn5* mRNA relative expression levels were observed during the first 2 weeks of CA, whereas differences in the accumulation of DHN5 protein between the growth habits, as well as between individual cultivars, gradually emerged during the same treatment. Moreover, the amount of accumulated DHN5 protein exhibited quantitative differences between the growth habits, with intermediate and winter cultivars having accumulated higher amounts of DHN5 than the spring ones, as well as between individual cultivars differing in their FT. A linear correlation between the amount of accumulated DHN5 protein and the acquired level of FT was obtained on 21 cultivars of all three growth habits, with relatively large differences in FT, after 3 weeks of CA. Thus, it can be concluded that DHN5 protein can be regarded as a marker of FT, but only if relatively large sets of various barley cultivars with contrasting levels of FT are used for analysis.

Acknowledgments

The authors thank Prof. T.J. Close from the Department of Botany and Plant Sciences, University of California, Riverside, CA, USA, for his generous gift of anti-dehydrin antibody. They also wish to thank Dr. H. Štorchová from the Institute of Experimental Botany, Czech Academy of Science, for consultations on the qRT-PCR analysis. The authors also would like to thank the anonymous reviewers for helpful comments on the manuscript. The work was supported by Grants MZe QF3191, MZe1G57060 and MZe0002700602.

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