Relationships between vernalization, frost tolerance and expression of dehydrins in barley (*Hordeum vulgare* L.)

Summary of PhD thesis

RNDr. Klára Kosová

Supervisor: RNDr. Ilja Tom Prášil, CSc.

Department of Plant Physiology, Faculty of Science, Charles University in Prague

Department of Genetics and Plant Breeding, Crop Research Institute, Prague - Ruzyně

2008
Declaration

I confirm that I obtained the results published in this thesis in my own experiments except the cases that are mentioned in the thesis. I declare that I have not used any part of this thesis in order to obtain any other academic degree.

In Prague, 25th September, 2008                                             Klára Kosová

Acknowledgement

First, I would like to thank my supervisor, RNDr. Ilja Tom Prášil, CSc., for his constant care, liberal kind of leadership and fruitful suggestions during my whole PhD study. I would also like to thank all members of our laboratory - Ing. Pavla Prášilová for practical advice about cultivation of plant material, Mgr. Pavel Vitámvás, PhD. for his practical advice about electrophoresis and other techniques of protein analysis, Ing. Eva Vlasáková and Ing. Zbyněk Škodáček for their good mood and Mgr. Lucie Davidová for her careful technical assistance with cultivation of plant material.
I also thank RNDr. Ludmila Holková from Department of Crop Science, Breeding and Plant Medicine, Faculty of Agronomy, Mendel University of Agriculture and Forestry, for fruitful collaboration and determination of dehydrin transcripts by qPCR analysis. I also have to thank RNDr. Věra Čapková, CSc., from the Institute of Experimental Botany, Czech Academy of Science, for helpful comments on SDS-PAGE and immunoblot techniques. I am very indebted also to Prof. Timothy J. Close from Department of Botany and Plant Sciences, University of California at Riverside, the U.S.A., for his generosity and his kind gift of anti-dehydrin primary antibody.

Last, but not at least, I have to thank my parents Jana and Milan, my sister Květa and my friend Zdeněk for their support during the whole study.

The experimental work was done in the laboratory of plant stress physiology at Department of Genetics and Plant Breeding, Crop Research Institute, Prague-Ruzyně. The work was supported by grants COST FA 0603, GA CR 522/08/1290, MZe 0002700602, MZe QF3191 and MZe 1G57060.
Contents

Introduction 1

Aims of PhD thesis 3

Results and Discussion 4

1. Evaluation of the possibility of the use of cold-induced dehydrin proteins for distinguishing barley cultivars with different level of frost tolerance 4

2. The dynamics of the individual development, the level of frost tolerance and DHN5 accumulation during a long-term CA treatment 5

2.1. The initial stages of CA treatment (0 - 14 days of CA) 5

2.2. The later stages of CA treatment (14 - 112 days of CA) 7

3. The relationship between the accumulation of DHN5 protein and the acquired level of frost tolerance 12

Conclusions 14

References 16

List of papers 18

Included manuscripts 20

Paper 1 (Abstract) 21

Paper 2 (Abstract) 22

Paper 3 (Abstract) 23

Paper 4 (Abstract) 24
Introduction

Barley (*Hordeum vulgare* L.), besides common wheat (*Triticum aestivum* (L.) em Thell.), is one of the most economically important cereal crops grown in temperate climate habitats including the territory of the Czech Republic. Despite mild winters in recent years, damage caused by frost still presents a serious threat to barley cultivation in temperate climate zones. Additionally, winter barley cultivars are generally more susceptible to frost than the winter wheat, rye and triticale cultivars (Fowler and Carles, 1979; Fowler, 2008). Therefore, a selection of sufficiently frost-tolerant barley cultivars is highly desirable and frost tolerance (FrT) as the ability to survive frost (temperatures below the freezing point) is one of key characteristics evaluated in barley breeding programmes.

In recent years, an immense boom in techniques of both structural genomics and functional genomics (*i.e.*, transcriptomics, proteomics and metabolomics) has provided a wide range of efficient tools usable in the breeding programmes. These techniques have enabled us the identification of specific DNA markers associated with the desired traits whose use in the breeding has launched a steep rise of techniques of marker-assisted selection (MAS). These markers can either be molecular (*i.e.*, a unique DNA sequence which may indicate a presence of a specific allele, but is not tightly linked to this allele; these markers are used in techniques of RFLP, AFLP, RAPD, SCAR, SNP, SSR, STS and others) or they can be functional (also called diagnostic or perfect markers), *i.e.*, an unique DNA sequence completely linked to a specific trait locus (Andersen and Lübberstedt, 2003). From an angle of view of functional genomics, the level of expression of a certain transcript (mRNA) or protein can correspond quantitatively to the acquired level of a trait of interest, thus the expression level of either an mRNA or a protein can also be considered a marker (see *e.g.*, Houde *et al.*, 1992).

FrT is not a stable characteristics of many frost-tolerant plants including cereals. Under optimum growth temperatures, the level of acquired FrT is usually quite low in both frost-sensitive and frost-tolerant plants. However, under the conditions of low, above-zero temperatures (cold; usually defined as temperatures below 12 °C or 10 °C), the level of acquired FrT rises rapidly in the frost-tolerant plants (Sakai and Larcher, 1985; Guy, 1990; Thomashow, 1999). Since FrT is a quantitative multigenic trait, it cannot be associated with a presence or an absence of a single allele whose presence or absence in the plant genome can easily be detected by a molecular marker.

Recently, it has been found out that the changes in FrT upon the conditions of cold acclimation (CA; the effect of a long-term cold treatment on a plant) correspond quite well to
the changes in expression and accumulation of some cold-inducible structural proteins. One important group of these proteins is a large family of COR/LEA proteins whose accumulation is associated with cellular dehydration. Cellular dehydration is an important component of some physiological processes - e.g., embryo maturation - as well as a result of the impact of many environmental stress factors including cold and frost. Therefore, one important group of COR/LEA proteins are LEA II proteins called also dehydrins.

In 1992, Houde et al. have identified a cold-inducible dehydrin protein, the WCS120 protein, in common wheat, and proposed this protein a potential marker of FrT in wheat. This hypothesis has been validated in our laboratory by Vitámvás et al. (2007) who distinguished two differently frost-tolerant cultivars of winter wheat on the basis of different level of accumulation of the WCS120 protein (family) (for review on Wcs120 gene family in common wheat, see Sarhan et al., 1997). In barley, an orthologue of the wheat Wcs120 gene named Dhn5 has been identified by Close et al. (1995) and its accumulation under cold has been proven by many researchers (Van Zee et al., 1995; Bravo et al., 1999; Choi et al., 1999; Zhu et al., 2000). However, no clear relationship between the accumulation of DHN5 protein and the acquired FrT level has been found in barley. Therefore, the study of the relationships between the development of FrT and the accumulation of DHN5 protein under cold acclimation in barley has presented the aim of my Ph.D. thesis.
Aims of PhD thesis

The main aim was to investigate the relationships between development (vernalization), frost tolerance (FrT) and accumulation of dehydrins (COR proteins) under cold acclimation conditions in barley.

To resolve these relationships, I postulated more specific aims:

- To get knowledge on the relationships between the regulation of development and frost tolerance under the conditions of cold acclimation in barley and in wheat as barley’s close relative (Paper 1).

- To get knowledge on the roles of dehydrins in plant cold acclimation process (Paper 2).

- To detect dehydrins induced by cold in barley and to choose a method for quantification of their accumulation at protein level. To evaluate the possibility of the use of DHN5 protein for differentiation among cultivars with different FrT level.

- To investigate the dynamics of frost tolerance development and the dynamics of $Dhn5$ gene expression and its protein product accumulation in a set of barley cultivars of different geographical origin and belonging to the three growth habits – intermediate, winter and spring (Paper 3).

- To investigate whether a relationship between DHN5 accumulation and acquired frost tolerance can be found in barley when high frost tolerance level is reached (Paper 3).

- To investigate the dynamics of frost tolerance development and DHN5 accumulation in Atlas 68 (a spring cultivar) and Igri (a winter cultivar) during a long-term CA (Paper 4).

- To investigate the relationship between developmental transition from the vegetative stage into the reproductive stage and the dynamics of acquired frost tolerance and DHN5 accumulation in Atlas 68 and Igri (Paper 4).

- To investigate the relationship between growth habit, acquired frost tolerance and accumulation of DHN5 protein in selected doubled haploid lines derived from Atlas 68 × Igri cross (Paper 4).
Results and discussion

1. Evaluation of the possibility of the use of cold-induced dehydrin proteins for distinguishing barley cultivars with different level of frost tolerance

Based on the data from literature, we examined the possibility of the use of dehydrin proteins for distinguishing barley cultivars with different level of FrT. On transcript level, expression of \textit{Dhn}5, \textit{Dhn}8 and \textit{Dhn}11 genes was reported in cold-treated barley plants (Choi \textit{et al.}, 1999; Zhu \textit{et al.}, 2000). On protein level, accumulation of one major dehydrin protein with relatively high molecular weight - the DHN5 protein (molecular weight according to sequence: 66.5 kDa; molecular weight according to electrophoretic mobility: 86 - 90 kDa) - was reported (Van Zee \textit{et al.}, 1995; Bravo \textit{et al.}, 1999; Zhu \textit{et al.}, 2000). Whereas Van Zee \textit{et al.} (1995) did not find any quantitative differences in the accumulation of DHN5 protein between frost-sensitive spring barley Morex and frost-tolerant facultative barley Dicktoo under CA conditions on immunoblots, Zhu \textit{et al.} (2000) found significant differences in DHN5 accumulation between Morex and Dicktoo under CA using the same immunoblot technique. Therefore, I first examined the possibility of the use of DHN5 or other dehydrin protein for distinguishing barley cultivars (lines) with different level of acquired FrT. I compared the profiles of heat-stable proteins in Atlas 68, a frost-sensitive spring barley cultivar, and in Igri, a relatively frost-tolerant winter barley cultivar, grown under CA conditions and found differences in DHN5 accumulation. I confirmed the identity of the spot on immunoblot. Therefore, I was able to conclude that DHN5 protein can be used for distinguishing barley cultivars with different level of acquired FrT. Since I worked only with one protein, I used the technique of 1D SDS-PAGE combined with immunoblots to compare DHN5 accumulation in different samples (Laemmli, 1970). For detection of DHN5 protein, I used a polyclonal primary antibody raised against dehydrin K-segment (Close \textit{et al.}, 1993).

The acquired FrT level was determined as LT\textsubscript{50} values, \textit{i.e.}, temperature when 50 % of the sample die, by a direct frost test according to Janáček and Prášil (1991) and Prášil and Zámečník (1998).
2. The dynamics of the individual development, the level of frost tolerance and DHN5 accumulation during a long-term CA treatment

2.1. The initial stages of CA treatment (0 - 14 days of CA)

In the initial stages of CA treatment (0 - 14 days of CA), expression of *Dhn5* gene, accumulation of the corresponding protein product and the development of FrT were monitored simultaneously on a set of twenty-one barley cultivars representing all growth habits - intermediate (syn. alternative, facultative), spring and winter (see Table 1).

The expression of *Dhn5* gene was determined by dr. Holková at Mendel University of Agriculture and Forestry in Brno by qPCR analysis. *Dhn5* gene is not expressed in the leaf tissues of barley grown under optimum growth temperatures (20 °C). After the exposure to CA, *Dhn5* gene becomes expressed in all barley cultivars belonging to different growth habits and the corresponding transcripts become detectable. The quantitative expression of *Dhn5* mRNA exhibits a typical curve in all growth habits during the first 14 days of CA. First, the expression of *Dhn5* rapidly increases until it reaches the maximum at 3 - 7 days of CA. After the initial burst, there is a significant drop in *Dhn5* expression in the subsequent period of CA which leads to the establishment of a low steady-state expression *Dhn5* level in the later stages of CA. No statistically significant differences in the *Dhn5* expression level between individual growth habits were observed (Fig. 1A).

DHN5 protein is not present in the leaf tissues of barley grown under optimum growth temperatures (20 °C). After the exposure to CA, DHN5 protein becomes accumulated in all
growth habits. In the initial phase of development, the accumulation of DHN5 protein rises in all growth habits, but with the length of CA treatment, differences in the level of DHN5 accumulation between individual cultivars begin occurring. The amount of accumulated DHN5 protein ceased increasing much earlier in the spring cultivars when compared with the winter and intermediate ones. In the winter and intermediate cultivars, the level of DHN5 protein continues rising at 14 days of CA (Fig. 1B).

Differences in DHN5 protein accumulation between a frost-sensitive spring barley cultivar Morex and a frost-tolerant facultative barley cultivar Dicktoo during the first 14 days of CA have already been described by Zhu et al. (2000).

After the exposure of the plants to CA treatment (3 °C), the level of FrT becomes increasing (i.e., the LT50 values become decreasing) in the barley cultivars belonging to different growth habits (intermediate, winter, spring). The initial decrease in LT50 values is rapid in all growth habits. However, with the progress of CA treatment, differences between individual growth habits began occurring. The LT50 values in the spring cultivars ceased decreasing much earlier than in the winter and intermediate ones which continued decreasing their LT50 values with the progress of CA (Fig. 1C).

These results, i.e., the rapid increase in acquired FrT level, Dhn5 transcript and DHN5 protein level in all growth habits at the beginning of CA treatment and the subsequent slowing down in the rate of FrT increase and DHN5 accumulation in the spring cultivars with respect to the winter ones, are in accordance with observations obtained in analogous studies on winter and spring wheat cultivars (see e.g., Danyluk et al., 2003; Kane et al., 2005; Ganeshan et al., 2008). These differences in the dynamics of FrT development can be explained by the results obtained by Monroy et al. (2007) in a transcriptomic study on a winter and a spring wheat. These researchers have found out that CA led to the initial burst of cold-inducible transcripts in both cultivars, but only the winter one was able to maintain enhanced levels of cold-inducible transcripts with the progress of CA treatment. In our experiments, however, we observed this pattern only at FrT level and DHN5 protein level, but not at Dhn5 transcript level. It should be considered that patterns of protein expression and gene expression do not always correspond and that the phenotypic traits are determined more strongly by proteins than by transcripts, i.e., the level of DHN5 protein accumulation corresponds to the level of acquired FrT better than the level of Dhn5 gene expression does. Thus it is possible to find the differences between cultivars with different level of FrT only at DHN5 protein level, but not at Dhn5 transcript level.
Table 1. 21 barley cultivars, their geographical origin, growth habit, number of rows in an ear and their frost tolerance determined as LT50 values after a 3-week CA. The cultivars are ordered according to their LT50 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

<table>
<thead>
<tr>
<th>Cultivar</th>
<th>Abbreviation</th>
<th>Origin</th>
<th>Growth habit</th>
<th>Ear</th>
<th>LT50 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lunet</td>
<td>Ln</td>
<td>CZE</td>
<td>I</td>
<td>six-row</td>
<td>-15.6</td>
</tr>
<tr>
<td>Dicktoo</td>
<td>Dc</td>
<td>USA</td>
<td>I</td>
<td>six-row</td>
<td>-15.3</td>
</tr>
<tr>
<td>Luxor</td>
<td>Lx</td>
<td>CZE</td>
<td>W</td>
<td>six-row</td>
<td>-15.2</td>
</tr>
<tr>
<td>Okal</td>
<td>Ok</td>
<td>CZE</td>
<td>W</td>
<td>six-row</td>
<td>-15.1</td>
</tr>
<tr>
<td>Hutorok</td>
<td>Hu</td>
<td>RUS</td>
<td>W</td>
<td>six-row</td>
<td>-14.8</td>
</tr>
<tr>
<td>Tiffany</td>
<td>Ti</td>
<td>DEU</td>
<td>W</td>
<td>two-row</td>
<td>-14.5</td>
</tr>
<tr>
<td>Luran</td>
<td>Lr</td>
<td>CZE</td>
<td>W</td>
<td>six-row</td>
<td>-14.4</td>
</tr>
<tr>
<td>Kromir</td>
<td>Ko</td>
<td>CZE</td>
<td>I</td>
<td>six-row</td>
<td>-14.3</td>
</tr>
<tr>
<td>Kromoz</td>
<td>Km</td>
<td>CZE</td>
<td>W</td>
<td>six-row</td>
<td>-14.3</td>
</tr>
<tr>
<td>Campill</td>
<td>Ca</td>
<td>DEU</td>
<td>W</td>
<td>six-row</td>
<td>-14.2</td>
</tr>
<tr>
<td>Vilna</td>
<td>Vi</td>
<td>NLD</td>
<td>W</td>
<td>two-row</td>
<td>-13.8</td>
</tr>
<tr>
<td>Jolante</td>
<td>Jl</td>
<td>DEU</td>
<td>W</td>
<td>two-row</td>
<td>-13.5</td>
</tr>
<tr>
<td>Igri</td>
<td>Ig</td>
<td>DEU</td>
<td>W</td>
<td>two-row</td>
<td>-13.4</td>
</tr>
<tr>
<td>Duet</td>
<td>Du</td>
<td>GBR</td>
<td>W</td>
<td>two-row</td>
<td>-13.1</td>
</tr>
<tr>
<td>Atlas 68</td>
<td>At</td>
<td>USA</td>
<td>S</td>
<td>six-row</td>
<td>-11.5</td>
</tr>
<tr>
<td>Prestige</td>
<td>Pr</td>
<td>FRA</td>
<td>S</td>
<td>two-row</td>
<td>-11.2</td>
</tr>
<tr>
<td>Jotun</td>
<td>Jt</td>
<td>NOR</td>
<td>S</td>
<td>six-row</td>
<td>-11.0</td>
</tr>
<tr>
<td>Braemar</td>
<td>Br</td>
<td>GBR</td>
<td>S</td>
<td>two-row</td>
<td>-10.9</td>
</tr>
<tr>
<td>Diamant</td>
<td>Da</td>
<td>CZE</td>
<td>S</td>
<td>two-row</td>
<td>-10.9</td>
</tr>
<tr>
<td>Sebastian</td>
<td>Se</td>
<td>DNK</td>
<td>S</td>
<td>two-row</td>
<td>-10.9</td>
</tr>
<tr>
<td>Amulet</td>
<td>Am</td>
<td>CZE</td>
<td>S</td>
<td>two-row</td>
<td>-10.0</td>
</tr>
</tbody>
</table>

2.2. The later stages of CA treatment (14 - 112 days of CA)

These experiments were conducted on a six-rowed spring barley cultivar Atlas 68, a two-rowed winter barley cultivar Igri and a set of twenty-one doubled haploid (DH) lines exhibiting either any vernalization requirement (winter DH lines) or no vernalization requirement (spring DH lines).

The length of CA led to the significant decrease in the length of subsequent cultivation at optimum growth temperature prior to heading in Igri whereas it did not have any effect on the heading dates in Atlas 68. In Igri, the vernalization requirement was 63 days under the CA treatment at 3 °C and 12 h photoperiod (Fig. 2A). This period coincides with the appearance
of the double-ridge stage when the shoot morphology was evaluated. The double-ridge stage indicates the irreversible transition from the vegetative stage into the reproductive stage (Hay and Ellis, 1998). It has been found out by Danyluk et al. (2003) that the expression of the major vernalization gene VRN-1 which is necessary for the developmental transition (Shitsukawa et al., 2007) precedes the formation of the double-ridge structure in the shoot apex. The length of CA also led to the increase in acquired FrT level (decrease in LT$_{50}$ values) in both Atlas 68 and Igri (Fig. 2B). In the initial stage of CA, the rate of the decrease of LT$_{50}$ values was quite rapid when compared with the later stages of CA and no significant differences between Atlas 68 and Igri as well as between the spring DH lines and the winter DH lines (FrT determined as percentage of plant survival after a direct frost test) occurred. However, in the later stages of CA, Atlas 68 ceased decreasing LT$_{50}$ values while Igri continued decreasing LT$_{50}$ values for a much longer time. The minimum LT$_{50}$ value in Atlas 68 was significantly higher (around -13 °C) and was reached earlier (around 35 days of CA) than in Igri (the minimum LT$_{50}$ value in Igri was around -17 °C and was reached around 70 days of CA). After that time, the LT$_{50}$

![Fig. 2. Days to heading (A), acquired frost tolerance level determined as LT$_{50}$ values (B) and relative accumulation of DHNS protein (C) in Atlas 68 and Igri plants during 112 days of cold acclimation treatment. In (C), the sum of DHNS protein accumulation in Atlas 68 and Igri at all sampling dates was set to 100%. Data in (A) and (C) represent mean values from five repetitions (n = 5), vertical bars represent standard errors (SE). Asterisks (*) indicate the data in which statistically significant differences (P<0.01) between Atlas 68 and Igri were found (Kosová et al. – Paper 4).]
values slightly increased in Atlas 68, but they did not change significantly in Igri until the end of CA treatment (112 days of CA). Analogous results have been obtained on the DH lines (Fig. 3B) when the plant survival rate in the spring DH lines reached the maximum at 21 days of CA and it declined gradually with the progress of CA. In contrast, the plant survival rate determined in the winter DH lines was the lowest at 21 days of CA (at that time, no significant differences in the plant survival rate between the spring and winter DH lines were found) and it gradually increased during the subsequent period of CA. The plant survival rate in the winter DH lines reached its maximum value at 63 days of CA and remained high also at 84 days of CA.

The level of DHN5 accumulation increased in both Atlas 68 and Igri leaves during the first 25 days of CA treatment (Fig. 2C and Fig. 4). At 25 days of CA, Atlas 68 reached its maximum DHN5 accumulation which was followed by a significant drop (decline) in DHN5 content. Analogously to Atlas 68 and Igri, the relative DHN5 accumulation per line did not significantly differ between the spring DH lines and the winter DH lines at 21 days of CA (Fig. 3C). The DHN5 content in Atlas 68 then
remained low, relatively unchanged until the end of CA treatment (112 days). Contrary to Atlas 68, the DHN5 accumulation in Igri steadily rose until 63 days of CA (the fulfillment of vernalization requirement) when it reached its maximum value. Analogously to Atlas 68 and Igri, statistically significant differences in the relative DHN5 accumulation per line between spring and winter DH lines were found at the remaining sampling dates (42, 63 and 84 days of CA) (Fig. 3C and Fig. 5). After 63 days of CA, the DHN5 accumulation in Igri decreased slowly at 77 and 91 days of CA and it declined more rapidly at 112 days of CA. At 112 days of CA, the DHN5 content in Igri was low and similar to the DHN5 content in Atlas 68.

We have found out that the fulfillment of vernalization requirement leads to the decrease in DHN5 accumulation level. This result is in accordance with the developmental theory postulated by Fowler et al. (1999) who proposed the idea that the transition from the vegetative stage into reproductive stage leads to the decrease in the ability to induce FrT and to accumulate COR proteins under CA. However, we have observed that the winter barley plants were able to maintain enhanced FrT level for quite a long time (up to 7 weeks after the fulfillment of vernalization requirement) when they were exposed to a continuous CA treatment. The dynamics of DHN5 accumulation in Igri after the fulfillment of vernalization thus differs from the dynamics of acquired FrT level in the same plants since the DHN5 content in Igri decreased after vernalization while the acquired FrT level remained high after vernalization. These results have been validated on the winter DH lines. This discrepancy between the dynamics of dehydrin protein accumulation and acquired FrT level after the fulfillment of vernalization has already been observed by Vítámvás and Prášil (2008) on the DHN5 orthologue in common wheat, the WCS120 protein, in the winter wheat Mironovskaya 808 after the fulfillment of vernalization. This phenomenon indicates that the level of accumulation of dehydrin proteins may be considered a marker of acquired FrT only in those barley plants that are in the vegetative stage of development, but not in those plants that are already in the reproductive stage of
We can conclude that study of the effect of a long-term CA on a set of selected Atlas 68 × Igri doubled haploid lines has shown that growth habit and the level of acquired FrT and DHN5 accumulation in the initial phases of CA (0 - 21 days of CA) are partly independent, i.e., lines with a spring growth habit (spring-type Vrn-H1/Fr-H1 locus) and a relatively high acquired FrT can be obtained in this population, and vice versa. A possible explanation of these results can lie in the fact that there are other FrT QTLs in barley genome except for the major FrT QTL at the Vrn-H1/Fr-H1 region. The second major FrT QTL has been mapped to Fr-H2 locus which is found ca 25 - 30 cM proximal to the Vrn-H1/Fr-H1 locus at 5HL. It has been recently found out by some authors (Choi et al., 2002; Francia et al., 2004; Skinner et al., 2005) that at the Fr-H2 locus, a cluster of ca 12 HvCBF genes is located. HvCBF genes are important transcription factors which regulate the expression of many Cor/Lea genes, including dehydrins, so they have a profound effect on the plant capacity to develop FrT under CA. It has recently been found out by Badawi et al. (2007) in common wheat and by Stockinger et al. (2007) in cultivated barley that differently frost-tolerant winter and spring cultivars differ also in the level of expression of several CBF genes located at the Fr-2 locus. Moreover, the winter and spring cultivars do not differ only in the level of CBF expression under cold, but also in the level of constitutive expression of some CBF genes at optimum growth temperatures. Stockinger et al. (2007) have confirmed a strong impact of the Vrn-H1/Fr-H1 locus on the expression of several Fr-H2 -located HvCBF genes (especially HvCBF2 and HvCBF4). Knox et al. (2008) have even found an allelic variation in the sequence of one TmCBF gene, TmCBF12, located at central part of Fr-A”2 locus of einkorn
wheat. The researchers found out that the frost-sensitive spring line encodes a non-functional allele of \textit{TmCBF12} gene which cannot bind to CRT/DRE motifs in the promoters of \textit{Cor} genes.

Therefore, the results obtained on Atlas 68 × Igri doubled haploid lines can be explained by the existence of the differences in the nature of the \textit{Fr} loci other than the \textit{Vrn-H1/Fr-H1} locus in the doubled haploid population (especially in the nature of the \textit{Fr-H2} locus in differently frost-tolerant lines). However, even the relatively frost-tolerant spring lines do not reach FrT levels and DHN5 levels comparable with the winter lines and, moreover, they lose their high acquired FrT level and the high amount of accumulated DHN5 much earlier than the relatively low frost-tolerant winter lines. The possible explanation lies in the differences in plant development (different dynamics of the vegetative/reproductive transition) between winter and spring lines. So it can be concluded that the growth habit does not strongly affect the level of acquired FrT and DHN5 protein accumulation in the initial phase of CA (before vegetative/reproductive transition), but it does strongly affect the maintenance of increased FrT level and DHN5 protein level during the CA progress.

3. The relationship between the accumulation of DHN5 protein and the acquired level of frost tolerance

It has been known for a long time that the cold-inducible dehydrin protein WCS120 can be used as a marker of acquired FrT level in common wheat (Houde \textit{et al.}, 1992), \textit{i.e.}, the level of the accumulation of WCS120 protein quantitatively corresponds to the level of acquired FrT in wheat. In barley (\textit{H. vulgare}), the orthologue of the \textit{Wcs120} gene was identified (Close \textit{et al.}, 1995) and was named \textit{Dhn5} (Choi \textit{et al.}, 1999). It has been confirmed by many researchers (Van Zee \textit{et al.}, 1995; Bravo \textit{et al.}, 1999; Choi \textit{et al.}, 1999; Zhu \textit{et al.}, 2000) that the \textit{Dhn5} gene is expressed upon the conditions of CA and its protein product accumulates in different barley tissues under CA. However, no quantitative relationship between the level of DHN5 accumulation and the acquired FrT level was observed in a set barley cultivars exhibiting different level of FrT (Bravo \textit{et al.}, 1999).

In our experiment, we used twenty-one barley cultivars of different geographical origin and growth habit which exhibit different levels of maximum acquired FrT (\textbf{Table 1}). At 21 days of CA treatment, sufficiently high acquired FrT level was reached in the most cultivars (\textit{i.e.}, it was possible to distinguish differently frost-tolerant barley cultivars according to their acquired FrT). At this time point, the accumulation of DHN5 protein in the fully developed
leaf tissues was analysed and a statistically significant ($r = 0.9$) correlation between the quantitative accumulation of DHN5 protein and the acquired FrT level was found (Fig. 6). However, it should be noted that this correlation was obtained only when this quite large set of barley cultivars with contrasting levels of FrT (highly frost-tolerant intermediate and winter cultivars versus less frost-tolerant spring cultivars) was used for the evaluation. When only winter or only spring cultivars were used for the evaluation, no correlation between DHN5 accumulation and acquired FrT level was found.

As it has already been mentioned in section 2.2., the level of DHN5 accumulation and the acquired level of FrT under CA treatment correlate only in those barley plants that are in the vegetative stage. After the vegetative/reproductive transition, a discrepancy in the dynamics of DHN5 accumulation and acquired FrT level was found (i.e., the DHN5 level declines more rapidly than the FrT level). This discrepancy indicates that the use of DHN5 accumulation as a potential marker of acquired FrT ought to be restricted only to the plants in the vegetative stage of development.

Fig. 6. The relationship between DHN5 accumulation (mean values) and FT in 21 barley cultivars after 21 days of CA. Vertical bars indicate SE ($n = 4$). The level of DHN5 accumulation is expressed relatively in percents; 100 % = total amount of DHN5 in 21 cultivars. (Kosová et al. 2008 – Paper 3).
Conclusions

In this section, I summarize the main results that I have obtained in Paper 3 and Paper 4.

- Based on the knowledge from literature and my own experiments, I have found out that DHN5 protein can be used for distinguishing barley cultivars (lines) with different level of acquired frost tolerance (FrT). For detection of the differences in DHN5 accumulation, 1D SDS-PAGE followed by immunoblots appeared as the most suitable method.

- Cold leads to the induction of FrT, Dhn5 gene expression and DHN5 protein accumulation in barley cultivars of all growth habits – intermediate, winter and spring. During the progress of CA, differences in FrT and DHN5 accumulation between the cultivars belonging to different growth habits become evident, possibly as a consequence of the differences in phenological development. Spring cultivars which do not have vernalization are able to induce increased FrT level and DHN5 accumulation only transiently in contrast to the winter and intermediate ones (winter cultivars have vernalization requirement which postpones the developmental transition into the less frost-tolerant reproductive stage; intermediate cultivars do not have vernalization; however, the developmental transition into the reproductive stage in intermediate cultivars is strongly inhibited by short photoperiods).

- Accumulation of DHN5 protein is cold-inducible and the amount of accumulated DHN5 protein is different in differently frost-tolerant barley cultivars. The amount of accumulated DHN5 corresponds well with the level of acquired FrT in the stage when high FrT is reached in all cultivars (at 21 days of CA). However, a highly significant correlation between DHN5 accumulation and acquired FrT level found in Paper 3 \( (r = 0.9) \) can be obtained only when a relatively large set of cultivars with contrasting levels of FrT (i.e., spring cultivars vs. winter ones) and different genetic background is used for the evaluation.

- During cold, both spring cultivar Atlas 68 and winter cultivar Igri start increasing the acquired FrT level and accumulating DHN5 protein. However, within the progress of CA, significant differences in the dynamics of frost tolerance development and DHN5 accumulation between these two cultivars occur. Spring cultivar Atlas 68 starts decreasing the acquired FrT level and DHN5 accumulation significantly earlier (after 3 weeks of CA) than winter cultivar Igri (after 9 weeks of CA). The difference in the dynamics of FrT development between the two cultivars can be attributed to the different dynamics of phenological development (indicated by the development of shoot apices), i.e., Atlas 68 exhibits earlier transition into the reproductive stage of development (marked by a double-ridge formation in the apex) than Igri under the same conditions of CA.

- Based on the simultaneous determination of acquired FrT, DHN5 accumulation and phonological development of the shoot apex, I can conclude that the developmental transition into the reproductive stage (indicated by a double-ridge formation) precedes the decline in acquired FrT and DHN5 accumulation in both spring cultivar Atlas 68 and winter cultivar Igri.
We have also found out that the relationship between the accumulation of DHN5 protein and the acquired level of FrT is strongly dependent on the stage of plant phenological development. We have found a similarity in the dynamics of DHN5 accumulation and development of FrT when the plants were in the vegetative stage of development. However, after the vegetative/reproductive stage transition, the DHN5 level started decreasing quite rapidly, but the acquired FrT level remained high for a much longer time under continuous CA treatment. This discrepancy between the DHN5 level and FrT level can be explained by the fact that the FrT is a complex multigenic trait which is determined not only by DHN5 level (and other COR/LEA protein levels), but also by many other factors (e.g., the intracellular levels of compatible solutes, proline, abscisic acid, the activity of ROS scavenging enzymes, the fluidity of the membranes). Thus it can be concluded that the DHN5 level corresponds to the acquired FrT only in those barley plants that are in the vegetative stage. The discrepancy between the DHN5 level and the acquired FrT level in the plants after the vegetative/reproductive transition presents an important limitation of the use of DHN5 protein accumulation as a potential marker of acquired FrT level in barley, i.e., it becomes evident that DHN5 protein accumulation can be used as a marker of acquired FrT level only in those barley plants that are in the vegetative stage.

- Study of the effect of a long-term CA on a set of selected Atlas 68 × Igri doubled haploid lines has shown that growth habit and maximum acquired FrT and maximum DHN5 accumulation in the initial phases of CA (0 - 21 days of CA) are partly independent, i.e., lines with a spring growth habit (spring-type Vrn-H1/Fr-H1 locus) and a relatively high acquired FrT can be obtained in this population, and vice versa. A possible explanation of these results can lie in the fact that there are other FrT QTLs in barley genome except for the major FrT QTL at the Vrn-H1/Fr-H1 region. However, even the relatively highly frost-tolerant spring lines decrease their acquired FrT level and the amount of accumulated DHN5 earlier during the progress of CA than the relatively low frost-tolerant winter lines. A possible explanation lies in the differences in plant development (different dynamics of the vegetative/reproductive transition) between winter and spring lines. So it can be concluded that the growth habit does not strongly affect the level of acquired FrT and DHN5 protein accumulation in the initial phase of CA, but it does strongly affect the maintenance of increased FrT level and DHN5 protein level during the CA progress.
References


List of papers

Research papers


Reviews


Contributions in proceedings


Abstracts in proceedings


Manuscripts that are included in the thesis:


**Paper 4:** Kosová, K., Prášil, I.T., Prášilová, P., Vitámvás, P., Chrpová, J.: The development of frost tolerance and DHN5 protein accumulation in a set of barley (*Hordeum vulgare*) doubled haploid lines derived from Atlas 68 × Igrí cross during a long-term cold acclimation. Submitted to Environmental and Experimental Botany.
The relationship between vernalization- and photoperiodically-regulated genes and the development of frost tolerance in wheat and barley

Kosová, K., Prášil, I.T., Vitámvás, P.


Abstract

The review summarizes the level of current knowledge of impacts of vernalization and photoperiod on the induction and maintenance of frost tolerance (FrT) in wheat and barley. The phenomenon of vernalization is briefly described and the major vernalization (VRN) loci are characterised. Vernalization requirement and the three major growth habits of Triticeae (facultative, winter and spring) are defined on the basis of the two-locus VRN-2/VRN-1 epistatic model. Major photoperiodically regulated genes, which influence the transition to flowering, are characterised and their interactions with VRN genes are briefly discussed. The phenomenon of induction of FrT during the process of cold acclimation (CA) is described and the major cold-induced Cor/Lea genes are listed. Important regulatory mechanisms, i.e., CBF pathway, controlling the expression of Cor/Lea genes under cold, are discussed. The major loci affecting the development of FrT in Triticeae, the Fr loci, are characterised. In conclusion, current progress in this research field is summarized and new questions arising in the area are formulated.
Paper 2

The role of dehydrins in plant response to cold

Kosová, K., Vítámvás, P., Prášil, I.T.


Abstract

Dehydrins present a distinct biochemical group of late embryogenesis abundant (LEA) proteins characterised by the presence of a lysine-rich amino acid motif, the K-segment. They are highly hydrophilic, soluble upon boiling, and rich in glycine and polar amino acids. It is proposed that they can act as emulsifiers or chaperones in the cells, i.e., they protect proteins and membranes against unfavorable structural changes caused by dehydration. Cold usually precedes freezing in nature and induces many physiological and biochemical changes in the cells of freezing-tolerant plant species (cold-acclimation) that enable them to survive unfavorable conditions. It is demonstrated that the induction of dehydrin expression and their accumulation is an important part of this process in many dicotyledonous both herbaceous and woody species, and also in winter cultivars of cereals, especially wheat and barley. Some mechanisms are discussed which are proposed to be involved in regulation of dehydrin expression, i.e., endogenous content of abscisic acid, homologues of Arabidopsis C-repeat binding factor (CBF) transcriptional activators, the activity of vernalization genes and photoperiodic signals. At the end, we outline some new approaches emerging to the solution of complex mechanisms involved in plant cold-acclimation, especially methods of functional genomics that enable to observe simultaneously changes in the activity of many genes and proteins in one sample.
Expression of dehydrin 5 during the development of frost tolerance in barley
(Hordeum vulgare)

Kosová, K., Holková, L., Prášil, I.T., Prášilová, P., Bradáčová, M., Vitámvás, P., Čapková, V.


Abstract

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 ten barley cultivars of different growth habit and geographical origin. The activation of Dhn5 gene expression was determined by qRT PCR, the accumulation of DHN5 protein by protein gel blot analysis using a specific anti-dehydrin antibody and the actual level of FT by direct frost test. During the first two weeks of CA, there was a rapid increase in Dhn5 gene expression, DHN5 protein accumulation and FT in all cultivars examined. After two weeks of CA, the differences in DHN5 accumulation and in FT measured as lethal temperature (LT_{50}) were observed between the cultivars belonging to different growth habits: intermediate (I) and winter (W) cultivars showed a higher level of DHN5 accumulation and LT_{50} values than the spring (S) ones which exhibited a lower level of accumulated DHN5 and FT. In contrast, no differences between the cultivars belonging to different growth habits in Dhn5 mRNA accumulation have been found. After three weeks of CA, the differences in accumulated DHN5 and FT between the individual growth habits became evident as a consequence of different developmental regulation of FT. The amount of accumulated DHN5 corresponded well with the level of FT of individual cultivars. It can be concluded 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.
The development of frost tolerance and DHN5 protein accumulation in a set of barley (Hordeum vulgare) doubled haploid lines derived from Atlas 68 × Igri cross during a long-term cold acclimation

Kosová, K., Prášil, I.T., Prášilová, P., Vitámvás, P., Chrpová, J.

Submitted to Environmental and Experimental Botany

Abstract

The dynamics of a long-term cold acclimation (CA) process was studied in spring barley cultivar Atlas 68, winter barley cultivar Igri and a set of selected doubled haploid (DH) lines derived from Atlas 68 × Igri cross. The aim of the study was to evaluate the effect of the plant development on the ability to induce frost tolerance (FT) and to accumulate dehydrin 5 (DHN5) protein during a long-term CA. The developmental stage of the plants was determined according to the phenological development of shoot apex and by determination of the length of plant cultivation at optimum growth temperature after a certain period of CA which was necessary to heading. The level of acquired FT was determined by direct frost tests in laboratory freezers. The accumulation of DHN5 protein was evaluated by densitometric analysis of the protein gel blots. The CA treatment led to the induction of increased FT level and to the accumulation DHN5 protein in both spring and winter DH lines. However, with the progress of CA treatment, differences between spring and winter DH lines began to occur as the winter DH lines were able to maintain increased FT level and DHN5 level for a significantly longer time than the spring DH lines. The probable cause of this difference was the absence of vernalization in spring lines which led to the much earlier transition into the reproductive growth stage in the spring lines when compared with the winter ones. After the developmental transition into the reproductive stage, a significant decrease in DHN5 accumulation was found in all lines; however, the FT level remained high for quite a long time. This discrepancy between FT level and DHN5 level in barley plants after the developmental transition is discussed.