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

PhD thesis

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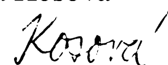
Declaration

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



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Souhrn

Disertační práce se zabývá studiem vlivu chladu na dynamiku vývoje mrazuvzdornosti u ječmene (*Hordeum vulgare* L.). V práci uvádím pokusy sledující vliv dlouhodobého chladového otužování na dynamiku vývoje mrazuvzdornosti u kultivarů zastupujících všechny tři růstové typy ječmene – jarní, ozimý a přesívka. K hodnocení vývoje mrazuvzdornosti jsem použila jak metody přímé (mrazové testy, konduktometrické stanovení poškození listových pletiv po mrazovém testu – Janáček a Prášil, 1991), tak i metody nepřímé - molekulárně-biologické - hodnocení akumulace chladově indukovaného proteinu DHN5 ze skupiny dehydrinů (COR/LEA proteiny) na imunoblotech s využitím primární protilátky proti K-segmentu jakožto konzervovanému sekvenčnímu motivu dehydrinů (Close *et al.*, 1993). Dehydrin DHN5 je nejvíce chladově-indukovaný COR protein u ječmene. Vliv chladu na individuální vývoj rostlin byl zjišťován jednak sledováním morfogeneze vzrostného vrcholu, jednak u ozimých kultivarů hodnocením vlivu chladu na zkracování jarovizačního požadavku rostlin (stanovován jako doba do metání po přenesení otužovaných rostlin do optimálních teplotních podmínek 25 °C).
První část disertační práce tvoří dvě literární review, z nichž první shrnuje poznatky o vztahu mezi individuálním vývojem pšenice a ječmene a jejich schopností indukovat mrazuvzdornost v podmínkách chladového otužování a druhé se zabývá úlohou dehydrinů při reakci rostlin na chlad. V experimentální části práce jsou uvedeny dvě publikace zabývající se tematikou vztahu individuálního vývoje (jarovizace), indukované mrazuvzdornosti a akumulace dehydrinů u různých odrůd ječmene.
Cílem disertační práce bylo sledování vlivu chladu na dynamiku vývoje mrazuvzdornosti u vybrané sady kultivarů náležejících k různým růstovým typům (jařiny, ozimy, přesívky) jednak pomocí přímého stanovení mrazuvzdornosti metodou mrazových testů, jednak pomocí hodnocení akumulace proteinu DHN5 (imunobloty). Dále jsem chtěla zjistit, zda lze využít míru akumulace dehydrinu DHN5 jako možný marker mrazuvzdornosti u ječmene (publikace 3).
V další fázi experimentů jsem se zabývala srovnáním dynamiky vývoje mrazuvzdornosti v průběhu dlouhodobého otužování (16 týdnů) u jarní odrůdy Atlas 68 a ozimé odrůdy Igri. Protože mě zajímaly vztahy mezi individuálním vývojem rostlin a jejich schopností indukovat mrazuvzdornost v podmínkách chladové aklimace, využila jsem možnosti pracovat se souborem vybraných dihaploidních linií vzešlých z křížení mezi odrůdami Atlas 68 a Igri. Pro svoji práci jsem si zvolila jednak linie jarní, bez jarovizace, avšak vykazující relativně vysokou míru indukované mrazuvzdornosti, jednak linie ozimé, mající jarovizaci, avšak vykazující jen mírnou mrazuvzdornost.
Ve své práci jsem zjistila, že po aplikaci chladu dochází u všech sledovaných odrůd patřících k různým růstovým typům (jařiny, ozimy, přesívky) k indukci mrazuvzdornosti i k indukci exprese dehydrinu *Dhn5*. V průběhu chladového otužování se však mezi jednotlivými růstovými typy postupně vytvářejí rozdíly – zatímco u ozimých odrůd mrazuvzdornost a akumulace dehydrinu DHN5 narůstaly (podstatě až do splnění jarovizačního požadavku), u jarních odrůd se nárůst mrazuvzdornosti i akumulace proteinu DHN5 poměrně brzy (zhruba po 3 týdnech otužování) zastavil a v pozdějších fázích otužování došlo naopak k poklesu mrazuvzdornosti i akumulace DHN5. V době dosažení vysoké indukované mrazuvzdornosti (po 3 týdnech otužování) jsem u souboru 21 odrůd zahrnujícího všechny růstové typy zjistila statisticky významnou korelaci mezi kvantitativní akumulací dehydrinu DHN5 a dosaženou úrovní mrazuvzdornosti. Je ovšem třeba v této souvislosti zdůraznit, že tuto korelaci jsem dostala především proto, že jsem pracovala s relativně velkým souborem odrůd s velmi rozdílnou dosahovanou úrovní mrazuvzdornosti (publikace 3).

Při porovnávání účinků dlouhodobého otužování na dynamiku vývoje mrazuvzdornosti u jarní odrůdy Atlas 68 a ozimé odrůdy Igri jsem zjistila, že v počátečních fázích otužování dochází u obou odrůd k výraznému zvýšení mrazuvzdornosti spojenému s indukcí exprese dehydrinu DHN5 ve srovnání s rostlinami pěstovanými v kontrolních podmínkách (ty se vyznačují jen relativně nízkou mrazuvzdorností a dehydrin DHN5 neexprimují). U jarní odrůdy Atlas 68 je však toto zvýšení mrazuvzdornosti vlivem chladu jen přechodné; po 3 týdnech otužování již dochází u odrůdy Atlas 68 k opětnému poklesu mrazuvzdornosti, zatímco u ozimé odrůdy Igri ve shodné fázi otužování mrazuvzdornost i akumulace proteinu DHN5 dále narůstají. U odrůdy Igri se mírný pokles mrazuvzdornosti začíná projevovat až po 9 týdnech otužování (po splnění jarovizačního požadavku). Dále jsem při sledování dynamiky individuálního vývoje a indukované mrazuvzdornosti u obou odrůd zjistila, že přechod do reproduktivního stadia vývoje (indikovaný tvorbou dvojitých hrbolků v oblasti vzrostného vrcholu) předchází pokles akumulace dehydrinu DHN5. Tento výsledek je ve shodě se závěry dalších autorů, kteří sledovali dynamiku individuálního vývoje (ať už sledováním morfogeneze vzrostného vrcholu či sledováním exprese genu *VRN-1*) a dynamiku vývoje mrazuvzdornosti či akumulace COR proteinů (Fowler *et al.*, 2001; Danyluk *et al.*, 2003).

Při práci s dihaploidními liniemi odvozenými z křížení Atlas 68 × Igri jsem zjistila, že růstový typ (jarní versus ozimé linie) nemá rozhodující vliv na míru akumulace proteinu DHN5 v počáteční fázi otužení (což lze vysvětlit existencí dalších QTL pro mrazuvzdornost kromě hlavního lokusu řídícího jarovizaci a mrazuvzdornost *Vrn-H1*/*Fr-H1*; dalším důležitým lokusem je např. *Fr-H2* lokus), avšak má významný vliv na dlouhodobé udržení (angl. maintenance) indukované mrazuvzdornosti. Jarní linie si nedokáží indukovanou mrazuvzdornost dlouhodobě udržet, ozimé linie ano; rozdíl zřejmě spočívá v dynamice jejich individuálního vývoje, kdy u jarních odrůd není přechod do reproduktivního stadia brzděn jarovizací, kdežto u ozimých odrůd je přechod do reproduktivního stadia možný až po splnění jarovizačního požadavku (publikace 4).

Zajímavým výsledkem bylo zjištění odlišností v dynamice vývoje mrazuvzdornosti a akumulace proteinu DHN5 u kultivaru Igri a u ozimých dihaploidních linií v pokročilejších vývojových stadiích (reproduktivní stadia vývoje po vytvoření dvojitých hrbolků) (též publikace 4). V tomto stadiu se u Igri i u ozimých dihaploidů projevil pokles akumulace proteinu DHN5, zatímco úroveň dosažené mrazuvzdornosti za nezměněných podmínek otužení výrazně neklesla. Obdobný rozdíl v dynamice akumulace dehydrinu a dosažené mrazuvzdornosti byl popsán již v práci Vítámvás a Prášil (2008) u rostlin ozimé pšenice Mironovskaya 808 po přechodu do reproduktivní fáze při pokusech se zpětnou reaklimací na chlad. Zjištěný rozdíl mezi úrovní akumulace dehydrinu a dosaženou úrovní mrazuvzdornosti ukazuje, že využití exprese proteinu DHN5 jako ukazatele (markeru) mrazuvzdornosti ječmene je omezeno jen na období vegetativního vývoje rostlin.

Citace:

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List of abbreviations

1D SDS-PAGE - one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis
3D - three-dimensional (structural model)
ABA - abscisic acid
AFLP - amplified fragment length polymorphism
AP - APETALA (gene)
CA - cold acclimation
CBF - C-REPEAT BINDING FACTOR (gene)
CD - circular dichroism
CO - CONSTANS (gene)
Cor - Cold-regulated (gene)
DH - doubled haploid (lines)
Dhn - dehydrin (gene)
DRE - DROUGHT-RESPONSIVE ELEMENT (promoter regulatory element)
DREB - DROUGHT-RESPONSIVE ELEMENT BINDING FACTOR (gene)
emf - embryonic flower (gene in *Arabidopsis thaliana*)
Eps - Earliness per se (locus)
ERD - EARLY RESPONSE TO DEHYDRATION (gene)
FLC - *FLOWERING LOCUS C* (gene in *Arabidopsis thaliana*)
Fr - Frost resistance locus
FrT - frost tolerance
FT - FLOWERING LOCUS T (gene)
IUPs - intrinsically unstructured proteins
LD - long-day (photoperiod)
LDH - lactate dehydrogenase
Lea - Late embryogenesis abundant (gene)
LT - low temperature (cold - temperatures above freezing point in the range of ca +12 - +10 °C - 0 °C; frost - temperatures below freezing point)
LT_{50} - lethal temperature when 50 % of the sample die (in frost tests)
LTRE - LOW TEMPERATURE RESPONSIVE ELEMENT (promoter regulatory element)
MAS - marker-assisted selection
mvp - maintained vegetative phase (mutant in *VRN-A^{m}1* gene in *Triticum monococcum*)
NLS - nuclear localisation signal
Ppd - photoperiod (locus)
PRR - PSEUDO-RESPONSE REGULATOR (gene)
qPCR - quantitative polymerase chain reaction (real-time PCR)
QTL - quantitative trait loci
RAPD - random amplified polymorphic DNA
RFLP - restriction fragment length polymorphism
ROS - reactive oxygen species
SCAR - sequence-characterised amplified region
SD - short-day (photoperiod)
SDS - sodium dodecyl sulfate
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SSR - simple sequence repeat
STS - sequence-tagged site
TF - transcription factor
UV - ultra-violet light (λ = 190 - 380 nm)

VIL - VERNALIZATION INSENSITIVE-LIKE
VIN - VERNALIZATION INSENSITIVE
VRN- VERNALIZATION (locus and corresponding gene)
VRT - VEGETATIVE-TO-REPRODUCTIVE TRANSITION (gene)
Wcs - Wheat cold-specific (gene)
ZCCT - ZINC FINGER CONSTANS CONSTANS-LIKE A

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 characteristic of many frost-tolerant plants including cereals. Under optimum growth temperatures, the level of acquired FrT is usually quite low in both frost-sensititve 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 Vítámvás *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). 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.

1. The regulation of frost tolerance development in *Triticeae*

Frost tolerance (FrT) is an important part of plant winter hardiness, *i.e.*, the ability of the plants to survive adverse winter conditions which include not only the tolerance to below-zero temperatures, but also desiccation tolerance, tolerance to the effects of snow cover or tolerance to flooding and to freeze-thaw cycles occurring regularly in some regions during the winter. FrT is defined as the ability of the plants to survive temperatures below zero (*i.e.*, frost). In most plant species including economically important cereals from the tribe *Triticeae*, FrT is not a stable characteristic of the plants, but it is a complex multigenic trait which is inducible upon the conditions of low, but above-zero temperatures. These temperatures induce profound physiological changes in plant tissues which are aimed at the elimination of adverse effects of low temperatures (LTs) (cold and frost). These complex processes are generally described as cold acclimation (CA) (Sakai and Larcher, 1985).

The LT stress shares some aspects with drought and osmotic stress. During freezing, the water in the cells would change into the ice crystals, thus becoming unavailable for the biomolecules inside the cells. In addition, the solid ice crystals would damage irreversibly the fine network of various intracellular structures. Thus the physiological processes in the plant cells subjected to CA are aimed at the decrease of intracellular water available for the formation and subsequent growth of ice crystal nuclei. During CA, profound changes in gene expression occur. The endogenous abscisic acid (ABA) level rises transiently during the initial phase of CA and many *de novo* proteins are synthesized. These proteins can have either catalytic (enzymes) or structural functions. The *de novo* synthesized enzymes catalyse changes in the saturation of fatty acids in the membranes in order to retain sufficient level of fluidity of the biomembranes under cold. Other enzymes scavenge ROS (reactive oxygen species), thus protecting the polyunsaturated chains of fatty acids from oxidation. Other groups of enzymes catalyse the synthesis of low-molecular hydrophilic compounds called osmolytes or compatible solutes which are of various chemical nature (low-molecular saccharides – monosaccharides glucose, disaccharides sucrose, oligosaccharides raffinose, stachyose and verbascose; imminoacid proline; polyamines spermine, spermidine, putrescine; quaternary ammonium compounds called betaines – *e.g.*, glycine betaine, alanine betaine; sugar alcohols like sorbitol, mannitol, pinitol) and which prevent the cell cytoplasm from intracellular freezing that can lead to water loss and denaturation of biomolecules (Guy, 1990).

The structural proteins have no enzymatic function; however, they also importantly contribute to the decrease in water loss during freezing. They are usually highly hydrophilic and can act as chaperones (*i.e.*, they can protect other proteins or other intracellular structures like membranes against unfavorable structural changes associated with excessive water loss which can result in the denaturation of these structures, *i.e.*, the loss of their biological activity). One important group of these proteins is a large family of COR/LEA (*Cold-regulated/Late embryogenesis abundant*) proteins (Ingram and Bartels, 1996; Thomashow, 1999).

Due to the profound physiological changes associated with the process of CA and the acquisition of FrT, the level of acquired FrT can be determined not only directly, but it can also be estimated indirectly from the measurements of the changes in water content, ABA content, osmotic potential or changes in *Cor* gene expression (Prášil *et al.*, 2007).

In the genome of *Triticeae*, several quantitative trait loci (QTLs) for FrT have already been mapped. The two major QTLs for FrT are called *Frost-resistance* loci (*Fr-1* and *Fr-2*) and

both are located on the long arm of homoeologous group 5 chromosomes (Cattivelli *et al.,* 2002; Francia *et al.*, 2004). The *Fr-1* locus is distal and it is tightly linked to the major vernalization locus *VRN-1* (Galiba *et al.*, 1995). It is becoming evident that these two loci cannot be clearly separated, so this chromosomal region is also described as *VRN-1/Fr-1* chromosomal region (Francia *et al.*, 2004; Stockinger *et al.*, 2007). The second *Frost-resistance* locus named *Fr-2* is about 25 – 50 cM proximal to the *VRN-1/Fr-1* locus and has been characterised as the main QTL affecting the expression of many *Cor/Lea* genes (Vágújfalvi *et al.* 2000, 2003). To the *Fr-2* locus, a cluster of *CBF* genes, whose homologues from *Arabidopsis thaliana* are known as the major activators of the expression of *Cor/Lea* genes (Jaglo-Ottosen *et al.*, 1998; Maruyama *et al.*, 2004; Vogel *et al.*, 2005), has been mapped (Choi *et al.*, 2002; Vágújfalvi *et al.*, 2003, 2005; Francia *et al.*, 2004; Skinner *et al.*, 2005; Miller *et al.*, 2006; Badawi *et al.*, 2007). The trans-activation of the expression of *Cor/Lea* genes by the *Triticeae'* homologues of *A. thaliana* CBF transcriptional activators has already been confirmed by gel shift assays (Xue, 2002, 2003; Skinner *et al.*, 2005). Zarka *et al.* (2003) have found out that in *A. thaliana*, the threshold induction temperature for the expression of *CBF* genes is around 14 °C.

The family of *CBF* transcriptional activators in *Triticeae* is much more numerous and diverse than in *A. thaliana.* (In *A. thaliana*, only four members of *CBF/DREB1* family have been described, three of them – *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A* - are cold-inducible and tandemly arranged on *A. thaliana* chromosome four (Gilmour *et al.*, 2004) while *CBF4/DREB1D* is drought- and ABA-inducible (Haake *et al.*, 2002).) In contrast, 13 *CBF* genes have already been mapped in the genome of einkorn wheat (*Triticum monococcum*) (Miller *et al.*, 2006), about 20 *CBF* genes are proposed for barley (*Hordeum vulgare*) (Skinner *et al.*, 2006), and at least 25 *CBF* genes are supposed to be found in the genome of common wheat (*Triticum aestivum*) (Badawi *et al.*, 2007). The diversification of the *CBF* gene family in *Triticeae* is attributed to the adaptation of these plants to temperate climate habitats with changing weather conditions. Most of these genes have already been mapped to the *Fr-2* locus in the *Triticeae'* genomes (Skinner *et al.*, 2005; Miller *et al.*, 2006; Badawi *et al.*, 2007) and the positions of the individual *CBF* genes at the *Fr-2* locus are currently being mapped precisely (Francia *et al.*, 2007; Knox *et al.*, 2008) in order to identify which members of the *CBF* cluster are responsible for the FrT QTL at this locus.

The nature of the *Frost-resistance locus 1* (*Fr-1*) remains unknown in all *Triticeae*; except for the major vernalization gene *VRN-1*, no other gene which would encode important

transcriptional regulator has been mapped to this chromosomal region. However, it has become evident that the *VRN-1/Fr-1* chromosomal region strongly affects the regulation of the expression of many *CBF* TFs located at the *Fr-2* locus and the expression of many *Cor/Lea* genes (see *e.g.*, Kobayashi *et al.*, 2005; Stockinger *et al.*, 2007) and one of the hypotheses which try to explain this phenomenon proposes that the FrT QTL at the *VRN-1/Fr-1* locus is a pleiotropic effect of the *VRN-1* gene itself (see *e.g.*, Reinheimer *et al.*, 2004; Stockinger *et al.*, 2007).

It has been known for a long time that the ability of *Triticeae* to induce increased level of FrT upon the conditions of CA is strongly affected by the plant developmental stage. The developmental theory formulated by Fowler *et al.* (1999) has postulated the statement that the transition from the vegetative to the reproductive stage of development, morphologically indicated by the formation of double ridges in the shoot apex of the plants indicates the irreversibility of the vegetative/reproductive transition (Nátrová and Jokeš, 1993; Hay and Ellis, 1998; Fowler and Limin, 2004) and it is generally accompanied by a loss in the plant ability to induce increased level of FrT and to accumulate increased levels of COR/LEA proteins upon CA. This means that the plants in the reproductive stage of their development can induce only a limited FrT level and accumulate a limited level of COR/LEA proteins under the conditions of CA when compared with the same plants in the vegetative stage of development (Fowler *et al.*, 2001; Prášil *et al.*, 2004; Ganeshan *et al.*, 2008; Vítámvás and Prášil, 2008). That is why the regulation of the developmental transition into the reproductive stage is so crucial for the plants. In *Triticeae*, the developmental transition is regulated by both internal (genetic) and external (environmental) factors. The internal developmental mechanisms are associated with several *Eps* (*earliness per se*) loci which may encode repressors of the premature transition to flowering functionally analogous to *A. thaliana emf* (*embryonic flower*) genes; however, candidate genes for *Eps* loci have not been identified yet (see *e.g.*, Laurie *et al.*, 1995; Laurie, 1997; Snape *et al.*, 2001; Bullrich *et al.*, 2002; Valárik *et al.*, 2006; Cockram *et al.*, 2007). The two most important environmental factors which strongly affect the developmental transition are vernalization and photoperiod.

Vernalization has been defined as the 'acquisition or acceleration of the ability to flower by a chilling treatment' (Chouard, 1960). Vernalization had evolved as an important regulatory mechanism which prevents the plants growing in higher latitudes or altitudes from a premature transition to flowering. Vernalization thus means a requirement of the plants to be grown under low temperatures for some period of time before gaining the

competence to flowering. The period of time for which the plants have to be grown under cold in order to gain the competence to flowering is called vernalization requirement. During the studies of vernalization in *A. thaliana*, it has been confirmed that the gain of the competence to flowering is associated with epigenetic modification of the activity of some genes, namely methylation of the chromatin region encoding the major flowering repressor *FLC*, which leads to the down-regulation of its gene expression (Bastow *et al.*, 2004; Sung and Amasino, 2004, 2005). In *Triticeae*, the epigenetic modifications during the developmental transitions have not been studied yet; however, some hypotheses about the epigenetic modification of the major vernalization gene *VRN-1* have already been formulated (Trevaskis *et al.*, 2007) and the homologues of the *A. thaliana VIN3* (*VERNALIZATION-INSENSITIVE 3*) and *VIL* (*VERNALIZATION INSENSITIVE3-LIKE*) genes from PHD finger family which are important components of the chromatin methylation machinery have already been identified by Fu *et al.* (2007) in the genome of einkorn wheat and the changes in their expression during vernalization have already been studied by the same authors. The plants can generally have either absolute or relative vernalization requirement. The plants with absolute vernalization requirement do not flower at all without any period of cold while the plants with relative vernalization requirement eventually flower without any period of cold, but the time of flowering is significantly delayed when compared to vernalized plants. Cereals from the tribe *Triticeae* do not have an absolute vernalization requirement, *i.e.*, all cultivars eventually flower without any period of cold, but there is a large difference in the time of flowering between unvernalized and vernalized plants in some cultivars.

In the genome of barley (*H. vulgare*), three major vernalization loci named *VRN-H1*, *VRN-H2* and *VRN-H3* have been mapped. All of them have already been characterised genetically (*i.e.*, it is known which gene is responsible for the effect of the locus). The major vernalization gene *VRN-H1* is located on 5HL and it encodes a MADS-box transcriptional regulator from the *AP1/SQUA* subfamily (Danyluk *et al.*, 2003; Yan *et al.*, 2003), *i.e.*, it encodes a transcriptional factor (TF) which is related to the *A. thaliana* flower meristem identity gene *AP1* (*APETALA1*). However, there are some differences in sequentional characteristics, expression pattern and functions between these two genes – for instance, *AP1* gene in *A. thaliana* is expressed only in the apical meristem, but *VRN-1* in *Triticeae* is expressed not only in the apical meristem before the formation of double-ridge structure (Danyluk *et al.*, 2003), but also in young leaves (see *e.g.*, Loukoianov *et al.*, 2005; Preston and Kellogg, 2007, 2008). It has been experimentally proven by Shitsukawa

et al. (2007) who have prepared a mutant of *T. monococcum* named '*maintained vegetative phase 1*' (*mvp1*) with impaired promoter and coding regions of the *VRN-A^{m}1* gene that the expression of the *VRN-1* gene is necessary for the transition to flowering. Analogously, Adam *et al.* (2007) have observed enhanced transition to flowering in transgenic *Arabidopsis* plants expressing the *TaVRN-1* gene from common wheat. This means that the *VRN-1* gene acts as a master 'switch' regulating the transition from the vegetative to the reproductive stage of development. Therefore, the regulation of the expression of *VRN-1* gene is highly important for the plant.

Two important regulators of the expression of the *VRN-1* gene are encoded by the other two *VRN* loci in barley. The *VRN-H2* locus is located on 4HL and encodes a transcriptional repressor of the *VRN-1* gene which is down-regulated by vernalization (fulfillment of vernalization requirement). Thus it is functionally analogous to *A. thaliana FLC* gene which is also the main repressor of flowering down-regulated by vernalization (Michaels and Amasino, 1999). However, the structure of these two transcriptional repressors is different – *FLC* belongs to a special sub-class of MADS-box genes found only in *Brassicaceae* family so far, but the *VRN-H2* candidate genes belong to the family of *ZCCT* TFs (*ZINC FINGER CONSTANS CONSTANS-LIKE AND TOC*) with a zinc-finger DNA binding domain encoded by the first exon and the *CCT* domain encoded by the second exon of the *ZCCT* gene. The *CCT* domains of the *ZCCT* genes are similar to the functional domains of *CO* (*CONSTANS*) and *PRR* (*PSEUDO-RESPONSE-REGULATOR*) genes (Yan *et al.*, 2004; von Zitzewitz *et al.*, 2005; Dubcovsky *et al.*, 2006). In *Triticeae*, only winter cereals have functional *VRN-2* genes and recessive alleles of *VRN-1* gene which can be repressed by functional *VRN-2* gene product. Thus only winter cereals do have vernalization requirement while the spring and facultative ones do not encode functional *VRN-2* genes, so they do not have any vernalization requirement (von Zitzewitz *et al.*, 2005; Szűcs *et al.*, 2007). It has been found out by the researchers (Dubcovsky *et al.*, 2006; Trevaskis *et al.*, 2006) that the expression of the *VRN-2* gene is also regulated by photoperiod besides low temperature. The expression of *VRN-2* genes is positively regulated (activated) by long-day (LD) photoperiod, but it is negatively regulated (repressed) by short-day (SD) photoperiod. This means that a sufficiently long period of SDs without any low temperatures (cold) leads to the down-regulation of *VRN-2* gene expression. This phenomenon is called 'short-day vernalization'. Some authors have also provided experimental evidence that a negative feedback loop between the expression of the *VRN-1* and *VRN-2* genes exists, *i.e.*, not only the *VRN-2* gene product represses the

expression of the *VRN-1* gene, but also the *VRN-1* gene, once being expressed, functions as a repressor of the *VRN-2* gene and leads to the down-regulation of the expression of the *VRN-2* gene product (Loukoianov *et al.*, 2005; Dubcovsky *et al.*, 2006).

However, it is well known that the *VRN-1* gene is expressed only under LDs, *i.e.*, it remains repressed under SDs even after the down-regulation of the *VRN-2* gene expression. Therefore, a hypothesis of at least one other repressor of *VRN-1* has been postulated. Recently, a SD-inducible transcriptional repressor *VRT-2* (*VEGETATIVE-TO-REPRODUCTIVE TRANSITION 2*) from the MADS-box family has been mapped to the short arm of homoeologous group 7 chromosomes and further characterised by Kane *et al.* (2005). These authors (Kane *et al.*, 2007) have already proven direct physical interaction between *TaVRT-2* TF and the CArG box (= a MADS-box TF binding motif in the promoter regions of many genes regulated by MADS-box TFs) in the promoter of *TaVRN-1* gene in an artificial system in *Nicotiana benthamiana.*

It has also been known for a long time that only the fulfillment of the vernalization requirement is not sufficient for the induction of the expression of *VRN-1*. The *VRN-1* gene needs a positive induction signal from a LD pathway. In the activation of *VRN-1* gene expression under LDs, the third vernalization gene in barley, the *VRN-H3* gene, is involved. The nature of this vernalization locus on 1HS has already been characterised by Yan *et al.* (2006) who found out that this locus encodes a transcriptional activator related to *FT* (*FLOWERING LOCUS T*) gene in *A. thaliana*. It is well known from *A. thaliana* that the *FT* gene also activates flowering under LDs in this plant and, recently, it has been confirmed by Corbesier *et al.* (2007) that the FT protein in *A. thaliana* is originally synthesized in the leaf tissue under LDs and then it is transported via floem to the plant apices (in accordance with the original hypothesis about florigen) where it interacts with a bZIP TF called FD. The FD protein binds to the promoter of *AP1* gene and the interaction between FT and FD proteins leads to the expression of the *AP1* gene in *A. thaliana.* Recently, analogous interaction between *Ta*FT1 protein (a protein encoded by *TaFT1* gene at *VRN-B3* locus in the genome of *T. aestivum*) and two homologues of *A. thaliana* FD protein in wheat (called *Ta*FDL2 and *Ta*FDL6) was described by Li and Dubcovsky (2008) in shoot apex meristem in wheat. The authors also found out that the *Ta*FT1 protein does not bind to the promoter region of the *VRN-1* gene directly, but it rather regulates the expression of the *VRN-1* gene indirectly via the interaction with *Ta*FDL2 protein which can bind to five ACGT elements in *VRN-1* promoter *in vitro*. The LD activation pathway in barley and wheat also includes genes from the families of *CO* and *PRR* genes which are

homologous to the *A. thaliana* genes of the same name (Putterill *et al.*, 1995; Griffiths *et al.*, 2003; Nemoto *et al.*, 2003; Turner *et al.*, 2005; Beales *et al.*, 2007) and which are known to be strongly involved in the photoperiodical regulation of flowering in *A. thaliana*.

In barley, two major *Photoperiod* (*Ppd*) loci named *Ppd-H1* and *Ppd-H2* affecting the transition to flowering were characterised (Laurie *et al.*, 1994, 1995). *Ppd-H1* locus has been mapped on 2HS and has been characterised as the principal inducer of flowering under LDs. *Ppd-H2* locus has been mapped on 1HL and has been characterised as the main repressor of flowering under SDs. Candidate genes for both *Ppd* loci have already been identified; *Ppd-H1* locus encodes a gene from *PRR* family (Turner *et al.*, 2005) and *Ppd-H2* locus encodes a gene from *FT* family (Faure *et al.*, 2007). Mutations in the *Ppd-1* loci in common wheat can result in photoperiodically insensitive lines flowering under both LDs and SDs (Beales *et al.*, 2007). The effect of the allelic constitution at *Ppd-H1* locus on the activation of *HvFT1* gene and the plant phenological development has recently been shown by Hemming *et al.* (2008) in barley.

Detailed information on the effects vernalization and photoperiod on the development of FrT under CA conditions in barley and common wheat is given in Paper 1.

2. The role of dehydrins in plant frost tolerance mechanisms

Dehydrins (the name is an abbreviation from 'dehydration-induced proteins') are an important group of COR/LEA proteins first characterised by Mundy and Chua (1988) in rice (*Oryza sativa*) and by Baker *et al.* (1988) in cotton (*Gossypium hirsutum*). They are also described as LEA II or LEA D-11 (according to the LEA proteins in cotton) proteins. (A very informative review summarizing the level of current knowledge about LEA proteins in plants has been recently published by Tunacliffe and Wise (2007).) Since their first characterization, dehydrins have been found in not only in all families of angiosperm plants studied so far, but also in gymnosperm plants, ferns, lycopods and mosses and dehydrin-like proteins have been found in various groups of algae (*e.g.*, in *Phaeophyceae*) and *Cyanobacteria*. [Recently, proteins related to LEA group have been found also in microorganisms and invertebrates which can survive drought in the state of anabiosis, *e.g.*, some nematodes (Browne *et al.*, 2002; Tunacliffe and Wise, 2007). Proteins distantly related to LEA group have even been described in vertebrates. In neurons, proteins known as α-synucleins are capable to form α-helices and associate with membranaceous vesicles (Ismail *et al.*, 1999; Souza *et al.*, 2000).] The accumulation of dehydrins and dehydrin-like

proteins confers predominantly osmotic stress tolerance in these organisms (Close and Lammers, 1993; Reynolds and Bewley, 1993; Close, 1996, 1997; Jarvis *et al.*, 1996; Li *et al.*, 1998; Velten and Oliver, 2001; Saavedra *et al.*, 2006). A positive effect of the expression of some dehydrin proteins on the improvement of salt, osmotic stress or frost tolerance has already been confirmed by transgenic studies (see *e.g.*, Hara *et al.*, 2003; Houde *et al.*, 2004; Puhakainen *et al.*, 2004; Yin *et al.*, 2006; Brini *et al.*, 2007).

2.1. Structure of dehydrins: All dehydrins are defined on the basis of their sequentional characteristics. All dehydrins share at least one copy of a conserved lysine-rich sequence (consensus **EKKGIMDKIKEKLPG**) which is usually located near the C-terminus of the molecule. Against this sequentional motif, primary antibody has been raised by Close *et al.* (1993) which is specific for the detection of dehydrins. This sequence can form a three-dimensional (3D) structure of class A2 amphipathic α-helix under the conditions of reduced hydration. The amphipathicity of the resulting 3D structure means that the hydrophobic amino acids like I and L lie on one side of the helix, the negatively charged amino acids like D and E lie on the opposite side of the helix, and the positively charged amino acids like K lie on the polar-nonpolar interface. Besides the K-segment, dehydrins also may (but not necessarily must) contain other conserved sequences: the Y-segment (a tyrosin-rich region; consensus **T/VDEYGNP**) usually located near the N-terminus, and S-segment (a stretch of 4 to 10 S) which is usually accompanied by other conserved sequence motifs (consensus $LHRSGS_{4-10}(E/D)_3$) which is proposed to be phosphorylated and to function as nuclear localisation signal (NLS) in its phosphorylated form (Close, 1996, 1997; Egerton-Warburton *et al.*, 1997, Svensson *et al.*, 2002). Besides these highly conserved regions, dehydrins also contain highly hydrophilic less conserved regions called Φ-segments (hydrophilic regions rich in G and polar amino acids S and T). In contrast, dehydrin molecules are usually absent from C (only in few dehydrin sequences known so far, C has been found and the formation of cystine bridges has been postulated – one of the few exceptions is the sequence of *Ppdhn3* gene from *Prunus persica* (Bassett *et al.*, 2006)). Dehydrins are also very poor in W. The whole dehydrin molecules are highly hydrophilic and remain soluble in aquaeous solutions even after boiling. This fact is often used for the enrichment of the samples with dehydrin proteins during the protein extraction process.

According to the arrangement of K-, S- and Y-segments in dehydrin molecules, five different structural sub-groups of dehydrins have been distinguished: K_n, SK_n, Y_nSK_n, K_nS and Y_nK_n. In all sub-groups, the K-segment is present at least in one copy per molecule (the maximum number of the copies of the K-segment is 11 copies per one molecule in CAP85

dehydrin protein from spinach (*Spinacia oleracea*) (Close, 1996, 1997; Campbell and Close, 1997). All angiosperm plants studied so far contain more than one dehydrin gene in their genome (*e.g.*, in the genome of *A. thaliana*, 6 dehydrin genes and 4 EST sequences have been characterised (Nylander *et al.*, 2001), in the genome of *H. vulgare*, 13 dehydrin genes have been found (Rodriguez *et al.*, 2005)). The dehydrin genes in one genome of an angiosperm plant usually belong to different structural sub-groups and are expressed under different conditions (Svensson *et al.*, 2002; Allagulova *et al.*, 2003; Rorat, 2006).

2.2. Expression of dehydrins: Dehydrin proteins can be found in low amounts in well-hydrated fast growing tissues of the root and shoot (described for dehydrins from wild potato (*Solanum sogarandinum*) by Rorat *et al.*, 2004, 2006), but they predominantly accumulate in plant cells under conditions of reduced hydration. These conditions include natural physiological processes such as embryo maturation and desiccation during the late stages of embryogenesis (hence the name of LEA proteins) as well as a wide range of abiotic stress factors such as drought, enhanced evaporation, increased salinity, cold and frost (Ingram and Bartels, 1996; for barley, see *e.g.*, Choi *et al.*, 1999; Suprunova *et al.*, 2004). There is also experimental evidence that some dehydrins can accumulate upon biotic stress of wounding, since wounding is usually accompanied by significant water loss. Accumulation of dehydrins upon wounding indicates the involvement of jasmonic acid and methyl jasmonate in the regulation of dehydrin expression (Reymond *et al.*, 2000; Richard *et al.*, 2000).

In plant cells, some dehydrin genes can be strictly embryo-specific (*e.g.*, *Dhn12* in barley; Choi and Close, 2000) while others can be predominantly cold-inducible, drought-inducible or can exhibit expression under a broad range of different abiotic stresses. These differences in dehydrin expression are determined by the presence of certain regulatory cis-elements in the promoters of various dehydrin genes which determine the activation of dehydrin expression by various signalling pathways. It is well known, for example, that ABRE regulatory elements to which bZIP transcriptional activators called AREBs or ABFs (ABRE binding factors) bind are crucial for the activation of the gene expression by ABA-dependent signalling pathways. Other ABA-dependent TFs interact with MYB and MYC regulatory elements in the promoters of many *Cor/Lea* genes. CRT/DRE/LTRE (C-repeat/drought-responsive elements/low temperature-responsive elements) are known as targets of cold-inducible CBF/DREB1 transcriptional activators as well as of predominantly drought-inducible DREB2 transcriptional activators which belong to ABA-independent signalling pathways (Stockinger *et al.*, 1997; Liu *et al.*, 1998). The resulting

composition of ABRE, CRT/DRE/LTRE, MYB and MYC elements in the promoter regions of dehydrin genes then determines the pattern of dehydrin expression under various environmental conditions (for review on abiotic stress signalling pathways and regulatory elements in the promoters of *Cor/Lea* genes, see *e.g.*, Bray, 1997; Zhu, 2002; Shinozaki and Yamaguchi-Shinozaki, 1997, 2000; Shinozaki *et al.*, 2003; Chinnusamy *et al.*, 2004, 2006; Yamaguchi-Shinozaki and Shinozaki, 2005, 2006; for original papers on this topic on dehydrins, see *e.g.*, Chung and Parish, 2008).

2.3. Function of dehydrins: As their name resembles, dehydrins are generally supposed to protect other proteins and membranaceous structures in cell cytoplasm and intracellular compartements from excessive water loss (see *e.g.*, Danyluk *et al.*, 1998; Wisniewski *et al.*, 1999; Koag *et al.*, 2003). (Dehydrins are exclusively intracellular proteins found in all cellular compartements including nucleus, plastids, mitochondria, vacuoles, endoplasmic reticulum; in contrast, no dehydrins have been detected in the cell wall; Egerton-Warburton *et al.*, 1997; Wisniewski *et al.*, 1999; Heyen *et al.*, 2002; Rorat, 2006.) Dehydrins thus function as intracellular chaperones. The basis of their chaperone function can be explained by the fact that dehydrin molecules are highly hydrophilic, *i.e.*, they can bind substantial amounts of water via hydrogen bonds. Under conditions of full hydration of the plant cells, the molecules of dehydrins have a conformation of random coil because they form many intermolecular hydrogen bonds between amino acid residues and the surrounding water molecules. During cellular dehydration, less water molecules are available for the formation of hydrogen bonds between the amino acid residues and the water, so intramolecular hydrogen bonds are becoming formed between the amino acid residues in one protein molecule and the conformation of the whole protein molecule is becoming changed. During this conformational change, the regions of the K-segments change their conformation from random coil to α-helix. (In random coil conformation, intermolecular hydrogen bonds between amino acid residues and water molecules are preferentially formed; in contrast, in α-helical conformation, intramolecular hydrogen bonds between two different amino acid residues are formed.) These α-helices then interact with partly dehydrated surfaces of other protein molecules or biomembranes and protect them from further dehydration.

Some authors (*e.g.*, Tunacliffe and Wise, 2007; Kovacs *et al.*, 2008) distinguish chaperone function as a specific interaction between dehydrin molecules and certain 'client' molecules from rather non-specific interactions between dehydrins and a wide range of various proteins and phospholipids during cellular dehydration. These non-specific

interactions can be explained by accumulation of dehydrins under cellular dehydration and their space-filling function during this process. It is becoming evident that the proteins accumulating as 'space-fillers' in the cell cytoplasm prevent aggregation of other proteins and membranes during cellular dehydration. These rather non-specific functions of dehydrins and other LEA proteins are often termed 'molecular shield'.

The conformational changes of dehydrin molecules during dehydration can be indirectly measured by the technique of far-UV CD (circular dichroism – the interaction of molecules with linearly polarized light). Random coil structures are relatively symmetrical three-dimensional (3D) structures, so they do not deviate the plane of linearly polarized light, while α-helices are highly asymmetrical 3D structures, so they significantly deviate the plane of linearly polarized light. The changes in far-UV CD spectra were really measured in the aquaeous solutions containing dehydrin molecules during the addition of detergents such as sodium dodecyl sulfate (SDS) by Danyluk *et al.* (1998), Ismail *et al.* (1999), and others. A detailed study on the conformational changes and chaperone activity of dehydrins has been recently published by Kovacs *et al.* (2008) on ERD10 and ERD14 proteins of *Arabidopsis thaliana*. The conformational changes which dehydrins as intrinsically unstructured proteins (IUPs; proteins with non-defined secondary structure under well-hydrated state) undergo during dehydration present a basis for the explanation of the phenomenon termed 'moonlighting' (Tompa, 2002; Tompa *et al.*, 2005), *i.e.*, the ability of some IUPs to carry out multiple functions.

Apart from this general function as intracellular chaperones or molecular shields, other specific stress-protective functions have been described in some dehydrins. A cryoprotective function, *i.e.*, the ability to protect the activity of some enzymes (usually determined by lactate dehydrogenase (LDH) assay) during a sudden drop of temperature (the drop in temperature can be even to the boiling point of liquid nitrogen 77 K) has been described by Neven *et al.* (1993) for CAP85 protein in spinach (*Spinacia oleracea*), Houde *et al.* (1995) for WCS120 protein in common wheat (*Triticum aestivum*), Wisniewski *et al.* (1999) for PCA60 protein in peach (*Prunus persica*), Hara *et al.* (2001) for CuCOR19 protein from Satsuma mandarin (*Citrus unshiu*), Bravo *et al.* (2003) for DHN5 protein in barley (*Hordeum vulgare*), and others. A positive effect on the activity of α-amylase under LT has been reported for a 24 kDa dehydrin from downy birch (*Betula pubescens*) by Rinne *et al.* (1999).

An antifreeze function, *i.e.*, a direct involvement in the formation and growth of ice nuclei (the modification of ice crystal growth and their resulting shape) has until now been described only for the PCA60 protein from peach (Wisniewski *et al.*, 1999).
Some dehydrin molecules also exhibit ROS (reactive oxygen species) scavenging functions which are carried out via direct chemical reactions between certain amino acid residues (namely H, K, R) and the ROS. ROS scavenging function has already been described for CuCOR19 protein from *Citrus unshiu* by Hara *et al.* (2004). It has also been found out that dehydrins can also participate in the reduction of ROS level in the cells indirectly since they can bind and thus inactivate free ions of heavy metals (Cu^{2+}, Fe^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Zn^{2+}) which generally act as important catalysers during the formation of ROS in cell cytoplasm. The chemical bonds between the ions of heavy metals and the dehydrin molecules are mediated by certain amino acid residues, namely H. One example of the dehydrin protein capable of binding substantial amounts of the ions of heavy metals (especially Cu^{2+}) is CuCOR15 isolated also from the flavedo tissue of *Citrus unshiu* by Hara *et al.* (2005). Recently, it has been found out by Alsheikh *et al.* (2003) that acidic dehydrins (= dehydrins with relatively low pI values) of the SK_n type – COR47, ERD10 and ERD14 - from *A. thaliana* undergo posttranslational modification – phosphorylation by cold-responsive protein kinases under cold. In their phosphorylated form, these proteins can bind substantial amounts of Ca^{2+} ions, thus the authors have hypothesized (Alsheikh *et al.*, 2005) that these dehydrins might be involved in cold-inducible Ca^{2+} signalling pathways. Phosphorylation-dependent calcium-binding activity has also been reported for a vacuole-associated dehydrin VCaB45 from celery (*Apium graveolens*) by Heyen *et al.* (2002).
It has also been known for a long time that the quantitative accumulation of some dehydrin proteins corresponds to the acquired level of frost tolerance (FrT) in some plants. A positive effect of the expression of some dehydrin proteins on the improvement of FrT has already been confirmed by transgenic studies (see *e.g.*, Houde *et al.*, 2004; Puhakainen *et al.*, 2004; Yin *et al.*, 2006). In common wheat (*T. aestivum*), this phenomenon has been described by Houde *et al.* (1992) for the cold-inducible accumulation of the WCS120 protein. The amount of the WCS120 protein which accumulates in different wheat tissues under cold can be thus used as a marker of acquired FrT level in wheat, *i.e.*, the more WCS120 protein the tissue has, the higher FrT level it exhibits. The WCS120 protein family (for review, see Sarhan *et al.*, 1997) includes exclusively cold-inducible, K_n type dehydrins encoded by chromosomal regions on the long arms of homoeologous group 6

chromosomes. Since the *Triticeae* genomes exhibit a significant degree of colinearity (*i.e.*, homologous genes can be found at homologous chromosomal positions), a K_n type homologue of the wheat WCS120 protein has been identified on 6HL in the barley genome by Close *et al.* (1995). This gene has been named *Dhn5* and it has been confirmed that this gene is also predominantly cold-inducible as its wheat orthologue (Van Zee *et al.*, 1995; Bravo *et al.*, 1999; Choi *et al.*, 1999; Zhu *et al.*, 2000). However, the researchers have observed the induction of *Dhn5* expression by cold, but they did not observe any correlation between quantitative accumulation of DHN5 protein and the level of acquired FrT in differently frost-tolerant barley cultivars.

Therefore, one of the major aims of my Ph.D. thesis was to investigate the possibility whether the DHN5 protein can be used as a marker of frost tolerance in barley analogously to its orthologue, the WCS120 protein in common wheat.

Detailed information on functions of dehydrin proteins in various plant species under cold is given in Paper 2.

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 *Dhn5*, *Dhn8* and *Dhn11* genes was reported in cold-treated barley plants (Choi *et al.*, 1999; Zhu *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 *et al.*, 1995; Bravo *et al.*, 1999; Zhu *et al.*, 2000). Whereas Van Zee *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 *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 *et al.*, 1993).

The acquired FrT level was determined as LT_{50} values, *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 in Paper 3).

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 in Paper 3).

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 in Paper 3).

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 LT_{50} values become decreasing) in the barley cultivars belonging to different growth habits (intermediate, winter, spring). The initial decrease in LT_{50} values is

rapid in all growth habits. However, with the progress of CA treatment, differences between individual growth habits began occurring. The LT_{50} values in the spring cultivars ceased decreasing much earlier than in the winter and intermediate ones which continued decreasing their LT_{50} values with the progress of CA (Fig. 1C in Paper 3).

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.

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. 1A in Paper 4). This period coincides with the appearance of the double-ridge stage when the shoot morphology was evaluated (Table 1 in Paper 4). 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. 1B in Paper 4). 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} 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 in Paper 4) 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. 1C and Fig. 2 in Paper 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. 4 in Paper 4). 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 development.

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.*, 2006) 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 $Fr\text{-}A^{m}2$ locus of einkorn wheat. The researchers found out that the frost-sensitive spring line encodes a non-functional allele of *TmCBF12* gene which cannot bind to CRT/DRE motifs in the promoters of *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 *Fr* loci other than the *Vrn-H1/Fr-H1* locus in the doubled haploid population (especially in the nature of the *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 *et al.*, 1992), *i.e.*, the level of the accumulation of WCS120 protein quantitatively corresponds to the level of acquired FrT in wheat. In barley (*H. vulgare*), the orthologue of the *Wcs120* gene was identified (Close *et al.*, 1995) and was named *Dhn5* (Choi *et al.*, 1999). It has been

confirmed by many researchers (Van Zee *et al.*, 1995; Bravo *et al.*, 1999; Choi *et al.*, 1999; Zhu *et al.*, 2000) that the *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 *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 (Table 1 in Paper 3). At 21 days of CA treatment, sufficiently high acquired FrT level was reached in the most cultivars (*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. 4 in Paper 3). 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.

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.

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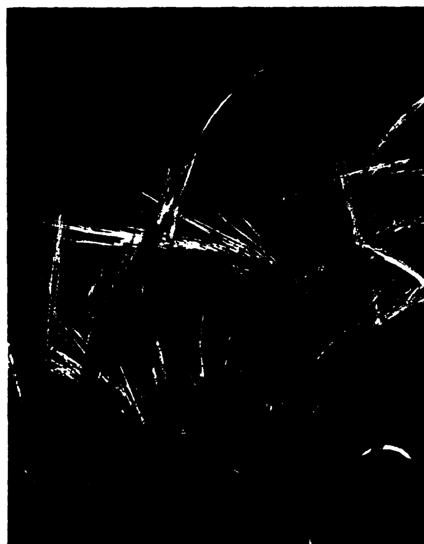

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REVIEW

The relationship between vernalization- and photoperiodically-regulated genes and the development of frost tolerance in wheat and barley

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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.

Additional key words: cold acclimation, *Hordeum*, *Triticum*.

Introduction

Low temperatures (LTs) not only induce a plant direct response *via* cold acclimation (CA), they also profoundly affect the plant developmental programme. Conversely, plant developmental stage has a crucial impact on a plant ability to cope with the unfavourable effects of LTs. The transition from vegetative phase into reproductive phase is generally associated with a decline in plant potential to resist the impacts of unfavourable environment. Plants originating from higher latitudes and/or altitudes had to adapt to relatively long periods of cold occurring regularly throughout the year. They thus had incorporated the requirement of a sufficiently long period of cold treatment into their individual developmental programme to prevent an early transition into the more cold-susceptible reproductive phase. This phenomenon, *i.e.*, the requirement of a sufficiently long cold period prior to the transition to flowering, is called vernalization. The process of CA is induced rapidly within a few days after the beginning of LT influence while the vernalization response develops for weeks and months of LT treatment. A comparison of the rates of plant responses to LT *via* CA and vernalization is given in Sung and Amasino (2004, 2005). In addition to cold, the developmental transition into the reproductive phase is regulated photoperiodically in some plants. In higher latitudes, the warm period of the year (summer) is associated with long

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Abbreviations: aa - amino acid; ABA - abscisic acid; *AP1* - *apetala 1* (gene); CA - cold acclimation; *CBF* - *C-repeat binding factor* (gene); *CO* - *constans* (gene); *Cor/Lea* - *cold-regulated/late embryogenesis abundant* (genes); *Dhn* - *dehydrin* (gene); *FLC* - *FLOWERING LOCUS C* (gene); *Fr* - *frost-resistance locus(i)*; FrT - frost tolerance; *FT* - *FLOWERING LOCUS T* (gene); *ICE-1* - *inducer of CBF expression 1* (gene); LD - long day; LT - low temperature; LT_{50} - lethal temperature when 50 % of the samples die; Mr - relative molecular mass; NIL - near isogenic line; pI - protein isoelectric point; *Ppd* - *photoperiod locus(i)*; *PRR* - *pseudo-response regulator* (gene); QTL - quantitative trait locus(i); ROS - reactive oxygen species; SD - short day; SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis; TF - transcription factor; *VRN* - *vernalization* (gene); *Vrn* - a dominant allele of *VRN* gene; *vrn* - a recessive allele of *VRN* gene; *Wcor* - *wheat cold-regulated* (gene); *Wcs* - *wheat cold-specific* (gene); *Wrab* - *wheat response-to-ABA* (gene)

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days (LDs) while unfavourable cold period of the year (winter) is connected with relatively short days (SDs). It became obvious that the photoperiod influences the expression of many regulatory genes including the genes which control the developmental transition into flowering.

The genera belonging to the tribe *Triticeae*, *i.e.*, wheat (*Triticum*), barley (*Hordeum*) and rye (*Secale*) are important cultivated cereals and form a homogeneous genetic system exhibiting colinearity of their genomes (*i.e.*, A, B and D genomes in cultivated wheat *T. aestivum*, H genome in *H. vulgare* and R genome in *S. cereale*). This means that genes found in one *Triticeae* genome have their counterparts located at the same chromosomal position in the other ones (apart from some exceptions caused by deletions, duplications, insertions or translocations, *e.g.*, different positions of *VRN-2* candidate genes in *T. monococcum* A^m genome and barley H genome). As a consequence of this important fact, the results obtained on one *Triticeae* member can be extrapolated to the others.

The aim of this review is to summarize the current state of knowledge of the connections between vernalization and photoperiod and the ability to induce a sufficient level of frost tolerance (FrT) in wheat and barley. In addition, some basic characteristics of wheat and barley vernalization loci, *Ppd* loci and photoperiodically-regulated genes, as well as cold-induced *Cor*/*Lea* genes are given in the form of tables.

The phenomenon of vernalization

Vernalization is an ecophysiological adaptation of plants originating from higher latitudes and/or altitudes which enables them to survive relatively long cold periods occurring regularly during a year. Vernalization has been rigorously defined as 'the acquisition or acceleration of the ability to flower by a chilling treatment' (Chouard 1960). Plants which require vernalization need exposure to a sufficiently long period of cold prior to 'switching' their individual developmental programme from the vegetative phase to the reproductive phase. Frost-tolerant plants in the vegetative phase can induce a sufficiently high level of FrT, while the same plants in the reproductive phase cannot since the generative organs (flowers and fruits) are generally more susceptible to cold in comparison with the vegetative ones (Sakai and Larcher 1985). Vernalization thus functions as an important control mechanism preventing the early transition into the less cold-tolerant reproductive phase. During vernalization, significant changes occur on the molecular level in the plants. In *Arabidopsis thaliana*, it was shown by Bastow *et al.* (2004) that vernalization leads to epigenetic silencing of the major flowering repressor *FLC via* histone methylation.

Winter cultivars belonging to the tribe *Triticeae* do not have an absolute requirement for vernalization, *i.e.*, they eventually flower without any period of cold; however, the time of flowering is significantly delayed (Krekule 1987).

Vernalization genes and growth habits

In wheat and barley, the existence of three vernalization loci has been shown. The loci have been named *VRN-1*, *VRN-2* and *VRN-3*. All vernalization loci encode transcription factors (TFs) affecting the regulation of other genes.

VRN-1 locus encodes the major vernalization gene which controls the transition to flowering. If the gene is active, *i.e.*, its gene product is present in the nucleus, profound changes in the expression of many TFs which eventually result in the formation of flower meristem are induced. The *VRN-1* gene can be present in the cereal genome in two types of alleles – repressible and irrepressible. The irrepressible allele *Vrn-1* is dominant, *i.e.*, if present in the genome, it has the major effect on plant development irrespective to the other allele(s). The repressible allele *vrn-1* can be inactivated by the binding of some TFs to either the CArG box in the promoter region (Yan *et al.* 2004a), or to a specific 436 bp 'vernalization critical region' in the first intron (Fu *et al.* 2005). It has been proposed that the CArG box in the promoter can function as a potential binding site for a MADS-box TF (Yan *et al.* 2004a). In contrast, the 'vernalization critical region' in the first intron is predicted to form four Dof sites which can be potential targets for a TF with a zinc-finger DNA binding motif (Von Zitzewitz *et al.* 2005). The irrepressible dominant *Vrn-1* allele has deletions in these critical regions (Fu *et al.* 2005). Thus, repressors cannot bind to them. Apart from the variability described above, Cockram *et al.* (2007a) have recently found out that some spring barley genotypes contain an insertion of a transposable element in the intron I region, upstream of the vernalization critical region. Analogously, an insertion of a transposon in the promoter region has been reported for the *VRN-B3* gene by Yan *et al.* (2006) (see below for more details). Thus, insertions of transposons seem to present another type of allelic variation in *VRN* loci.

The *VRN-1* gene encodes a MADS-box TF of the AP1 family. The same gene has also been named *HvBM5A* in barley (*H. vulgare*) (Von Zitzewitz *et al.* 2005), *TmAP1* gene in diploid wheat (*T. monococcum*) (Yan *et al.* 2003) and *WAP1* (Murai *et al.* 2003) and *TaVRT-1* (Danyluk *et al.* 2003) genes in hexaploid wheat (*T. aestivum*). The *VRN-1* gene is located on the long arm of chromosome 5. In barley genome, there is only one *VRN-1* gene located on 5HL; this gene is described as *VRN-H1*. Analogously,

in diploid wheat (*T. monococcum*), the only *VRN-1* gene is located on $5A^mL$ and described as *VRN-A^{m}1*. In hexaploid wheat (*T. aestivum*), there are three *VRN-1* genes described as *VRN-A1* on 5AL, *VRN-B1* on 5BL and *VRN-D1* on 5DL, respectively. While the dominant *Vrn-A1* allele completely reduces vernalization in hexaploid wheat, cultivars carrying only dominant *Vrn-B1* or *Vrn-D1* alleles usually show some residual vernalization requirement. This hypothesis has already been explained by Loukoianov *et al.* (2005) as differences in transcription: transcripts of dominant *Vrn-A1* allele appear earlier and in larger quantities in leaf tissue than transcripts of dominant *Vrn-B1* and *Vrn-D1* alleles during the plant development. The authors have also suggested a feedback loop in which winter alleles of *VRN-B1* and *VRN-D1* become induced without vernalization if a spring *Vrn-A1* allele is present in the genome of common wheat. This mechanism is probably mediated via a *Vrn-A1*-dependent downregulation of the expression of *VRN-2*. Moreover, the *Vrn-1* alleles are not completely dominant over the recessive *vrn-1* alleles at the same locus because it has been shown by some researchers (*e.g.*, Kóti *et al.* 2006) that heterozygotes head significantly later than *Vrn-H1/Vrn-H1* homozygotes in a barley cross between Hardy (winter) and Jubilant (spring) cultivars. Košner and Pánková (1998) have also hypothesized that an allelic variation in recessive *vrn-1* alleles may exist which could affect the length of vernalization in various winter wheat cultivars.

The *VRN-1* genes in *Triticeae* are closely related to *Arabidopsis* MADS-box genes of the *AP1/SQUA* subfamily, especially to the flower meristem identity genes *AP1* (*APETALA1*), *CAL* (*CAULIFLOWER*) and *FUL* (*FRUITFULL*) (Yan *et al.* 2003, Laurie *et al.* 2004). However, the *VRN-1* genes which are often described as *Arabidopsis AP1* homologues in the *Triticeae* differ from the *Arabidopsis AP1* gene in some significant aspects: they are sequentionally more related to *A. thaliana FUL* gene than to the *A. thaliana AP1* gene, they are expressed not only in the flower meristem (as is the case for *Arabidopsis AP1*), but also in some types of the vegetative tissue such as leaves. Moreover, their potential function in the formation of flower meristem and determination of floral organs (which is precisely described for *Arabidopsis AP1* gene – a flower meristem identity gene and a gene of the A-type function in the classical ABC model of flower development) still remains to be elucidated (Schmitz *et al.* 2000). Recent advances in the elucidation of the roles of *AP1/FUL* genes in the morphogenesis of floral meristems in *Poaceae* can be found in Preston and Kellogg (2007).

The *VRN-2* locus is located on chromosome 4HL in barley. However, in the A genome, the part of 4AL with the *VRN-2* locus had been translocated to $5A^mL$ (Cattivelli *et al.* 2002, Yan *et al.* 2004b). Thus, the *VRN-2* locus is found at $5A^mL$ in *T. monococcum*. The *VRN-2* locus in barley consists of three tightly linked genes *ZCCT-Ha*, *ZCCT-Hb* and *ZCCT-Hc* (Dubcovsky *et al.* 2005). In *T. monococcum*, two tightly linked genes named *ZCCT-1* and *ZCCT-2* have been identified at *VRN-2* locus (Yan *et al.* 2004b). *ZCCT-Ha* in barley and *ZCCT-1* in *T. monococcum* have been reported to be the most likely candidates for the *VRN-2* gene (Yan *et al.* 2004b, Dubcovsky *et al.* 2005). All these genes encode *ZCCT* (zinc-finger, *CONSTANS*, *CONSTANS*-like, and *TOC*) TFs which are down-regulated by vernalization. The *ZCCT* TFs contain two important binding domains, the C_2H_2 (2 cysteine 2 histidine) zinc-finger domain, which can bind both DNA and protein and which is encoded by the first exon, and the CCT domain, which controls nuclear localization of the TF (Robson *et al.* 2001) and binds to the CCAAT box binding factors, which mediate the interactions between *CONSTANS*-like proteins and DNA (Ben-Naim *et al.* 2006). The CCT domain is encoded by the second exon. The *VRN-2* gene can also be present in dominant (functional) and recessive (loss-of-function point mutation – *vrn-2a*; complete deletion – *vrn-2b*) alleles. The loss-of-function mutations in *ZCCT-1* are associated with a substitution of a single conserved arginine to a tryptophan at position 35 of the CCT domain which correlates with the spring growth habit in *T. monococcum* (Yan *et al.* 2004b).

In *T. aestivum*, the *VRN-2* loci have not yet been mapped nor further characterised due to the absence of trait variation. A possible explanation of the failure in mapping of the *VRN-2* loci in common wheat may lie in the fact that the recessive phenotype in *VRN-2* in hexaploid wheat would require complete deletion or loss-of-function mutation in the *ZCCT* genes in all three genomes. However, it can be expected that the winter cultivars of common wheat contain functional *ZCCT* genes analogously to the situation in *T. monococcum* and in barley; otherwise, Yan *et al.* (2004b) did not prove the role of the *ZCCT1* gene as a repressor of the *VRN-1* gene by an RNAi experiment in a winter cultivar of common wheat.

The *VRN-3* locus encodes a TF orthologous to *Arabidopsis FT* (*FLOWERING LOCUS T*) (Yan *et al.* 2006) and is located on chromosome 7HS in barley and 7BS in wheat, where it was formerly described as *VRN-B4* locus. The candidate genes have been named *HvFT1* in barley and *TaFT1* in wheat. The dominant allele enhances flowering *via* up-regulation of the *VRN-1* gene expression. In barley, the dominant allele has a deletion in its first intron with respect to the recessive one, whereas in wheat, the dominant *Vrn-3* allele has an insertion of a retroelement in the promoter in contrast to the recessive one. The dominant alleles in both wheat and barley enhance flowering under LDs as it does *FT* TF in *A. thaliana* and it is proposed that they are under regulation of *Ppd-1* locus (Fig. 1, Table 1).

Since no allelic variation at the *VRN-3* locus has been observed in most accessions belonging to cultivated wheat and barley, a two-gene epistatic model of vernalization considering *VRN-1* and *VRN-2* genes only has been proposed (Yan *et al.* 2003, Szűcs *et al.* 2007). *VRN-2* gene is a repressor of *VRN-1*, *i.e.*, it inhibits the expression of the *VRN-1* gene product. In this model, the

VRN-1 gene acts as the major developmental gene 'switching' the individual plant development from the vegetative phase to the reproductive phase. Cultivars carrying at least one dominant *Vrn-1* allele do not have any vernalization requirement. If the cultivars carry only recessive *vrn-1* alleles, these alleles can be repressed by the *VRN-2* gene product (Fu *et al.* 2005, Dubcovsky *et al.* 2006). The *VRN-2* gene can also be present in two alleles – dominant (functional repressor) and recessive (the repressor is absent or unfunctional). It has been shown by Yan *et al.* (2004b) that the *VRN-2* gene product is down-regulated by vernalization. The function of *VRN-2* as a repressor of *VRN-1* has been proposed by Yan *et al.* (2004b) on the basis of an RNAi experiment: the insertion of an RNAi segment complementary to the *ZCCT1* gene in the hexaploid winter wheat cultivar Jagger resulted in the up-regulation of *VRN-1* and reduction of the vernalization requirement. Thus, the winter growth habits which contain only recessive *vrn-1* alleles and at least one dominant *Vrn-2* allele do have a vernalization requirement. Later, it was shown by some researchers (Dubcovsky *et al.* 2006, Trevaskis *et al.* 2006) that this model is valid only under LD conditions, while SD conditions lead to the down-regulation of *VRN-2* expression regardless of the temperature which does not result in the up-regulation of *VRN-1* expression in *vrn-1vrn-1* homozygous genotypes. Therefore, the existence of at least one other repressor of *VRN-1*, which may repress its expression under SDs, has been postulated.

The *VRN-2* gene as a central repressor of the *VRN-1* gene down-regulated by vernalization has no clear orthologues in *Arabidopsis*. In *A. thaliana*, the central flowering repressor *FLC* is a MADS-box TF of a special subclass, which is not found in grasses, and analogously, *ZCCT* TFs, which are candidate genes for the *VRN-2* locus in *Triticeae*, have not been identified in the vernalization response pathway in *Arabidopsis* yet (Kane *et al.* 2005). Moreover, the CCT domains from the cereal *ZCCT* genes belong to a specific sub-group which does not occur in *Arabidopsis* (Yan *et al.* 2004b). These findings support the thesis that the vernalization regulatory pathways in *Arabidopsis* and cereals had developed independently.

Based on the *VRN-1* and *VRN-2* alleles and their interactions, three major growth habits have been defined in wheat and barley: the winter habit, which has vernalization requirement, the spring habit, which does not have vernalization, and the facultative habit, which also does not have vernalization, but is strongly photoperiodically sensitive. The spring habit has at least one dominant *Vrn-1* allele, *i.e.*, the transition to the reproductive phase cannot be repressed by vernalization. The facultative habit has the allelic constitution *vrn-2vrn-2*/*vrn-1vrn-1* which means that the *vrn-1* allele is not repressed by *vrn-2* (and thus the facultative growth habit does not have any vernalization requirement), but it can be repressed by other genes, *e.g.*, by photoperiodically activated TFs which cause that facultative habit is very photoperiodically sensitive. Therefore, the facultative growth habit remains in the vegetative phase unless stimulated to flower by LDs. The winter habit has the allelic constitution *Vrn-2_*/*vrn-1vrn-1* which means that it has a vernalization requirement to down-regulate the expression of the *Vrn-2* allele in order to de-repress the *vrn-1* allele (Von Zitzewitz *et al.* 2005, Szűcs *et al.* 2007).

The relationship between *VRN-1* and *VRN-2* genes is epistatic, *i.e.*, if a dominant *Vrn-1* allele is present in the genome, its presence results in a spring growth habit regardless of the allelic constitution at the *VRN-2* locus (dominant epistasis of *VRN-1* over *VRN-2*). It has been suggested by some authors (Loukoianov *et al.* 2005, Trevaskis *et al.* 2006) that the dominant *Vrn-1* allele could act as a repressor of the dominant *Vrn-2* alleles, *i.e.*, if a dominant *Vrn-1* allele is present in the genome, it could downregulate the expression of *Vrn-2*.

Most of the wild *Triticeae* belong to the winter growth habit. It seems to be unlikely that a vernalization requirement would have developed independently at the same locus in all *Triticeae*. Thus, it could be proposed that a winter growth habit and the recessive *vrn-1* allele are ancestral, and the dominant spring allele *Vrn-1* has evolved independently as a loss-of-function allele (loss of vernalization requirement) via mutations in its regulatory sites – the promoter CArG box and the 'vernalization critical region' in the first intron (Yan *et al.* 2003).

Table 1. The list of vernalization loci and their candidate genes characterised in barley (*H. vulgare*), in einkorn wheat (*T. monococcum*) and common wheat (*T. aestivum*).

Species	Locus	Gene - transcription factor	Location	Reference
H. vulgare	*VRN-H1* (formerly *Sh 2*)	*HvBM5A* – MADS-box	5HL	Von Zitzewitz *et al.* (2005)
	VRN-H2 (formerly *Sh*)	*ZCCT-Ha*, *ZCCT-Hb*, *ZCCT-Hc* – zinc-finger TF with CCT domain	4HL	Dubcovsky *et al.* (2005)
	VRN-H3 (formerly *Sh 3*)	*HvFT* - orthologue of *FT* in *A. thaliana*	7HS	Yan *et al.* (2006)
T. monococcum	*VRN-A^{m}1*	*TmAP1* – MADS box	5A^{m}L	Yan *et al.* (2003)
	VRN-A^{m}2	*ZCCT-1*, *ZCCT-2* (zinc-finger and CCT TF)	5A^{m}L	Yan *et al.* (2004b)
T. aestivum	*VRN-A1* (formerly *VRN1*)	*WAP1*- MADS-box	5AL	Murai *et al.* (2003)
	VRN-B1 (formerly *VRN2*)	*TaVRT-1*- MADS-box	5BL	Danyluk *et al.* (2003)
	VRN-D1 (formerly *VRN3*)		5DL	
	VRN-B3 (formerly *VRN-B4*)	*TaFT* - orthologue of *FT* in *A. thaliana*	7BS	Yan *et al.* (2006)

Photoperiodically-responsive loci and genes involved in the transition to flowering

Certain developmental processes in plants are initiated when the photoperiod (day-length) is either longer or shorter than a certain day-length which is called 'critical'. The critical photoperiod is not the same in all cases – it depends on the plant species and the developmental process it regulates. According to the effects of the photoperiod on the induction of flowering, plants can be divided into three major groups: long-day (LD) plants, short-day (SD) plants and neutral plants. LD plants flower when the day-length is longer than a certain critical photoperiod while SD plants flower when the day-length is shorter than a critical photoperiod. Neutral plants are photoperiodically insensitive, *i.e.*, they flower independently on the day-length. For photoperiod sensing, phytochrome photo-receptors are crucial.

Cereals belonging to the tribe *Triticeae* are photoperiodically-sensitive; the transition to flowering is promoted by LDs. In barley, the major photoperiodically-sensitive genes controlling the transition to flowering are located at two *Ppd* (*Photoperiod*) loci, *Ppd-H1* and *Ppd-H2* (Laurie 1997). The *Ppd-H1* locus is found on 2HS and is a principal inducer of flowering under LDs (Laurie *et al.* 1994, Karsai *et al.* 1997). The *Ppd-H2* locus is found on 1HL and is a principal repressor of flowering under SDs (Laurie *et al.* 1995). Previously, *CO*-like (*CONSTANS*-like) genes, photoperiodically regulated genes, which control the transition to flowering in *Arabidopsis* (Putterill *et al.* 1995), potato (González-Schain and Suárez-López 2008) and many other plants, have been proposed to be likely candidate genes for *Ppd* loci. Nine *CO*-like genes, *HvCO1* to *HvCO9*, have been identified in barley (Griffiths *et al.* 2003); however, they are not located at the *Ppd* loci in the barley genome.

Later, Turner *et al.* (2005) have identified a *ppd-H1* mutant of barley which exhibits a reduced photoperiodical responsiveness, reduced expression of *HvFT1*, and an altered circadian timing of *CO* expression. The *ppd-H1* mutation is associated with a substitution of a single conserved glycine residue to tryptophan in the CCT domain of a *PRR* (*PSEUDO-RESPONSE REGULATOR*) gene. *PRR* genes like *CO* genes are involved in the timing of the internal circadian clock or are involved in output pathways from the clock and both gene families share the CCT domain. Apart from the CCT domain, all *PRR* genes contain a pseudoreceiver domain with similarities to bacterial two-component signalling systems. It is well known from *Arabidopsis* that *PRR7* gene has a relatively strong influence on the expression of *FT* (Nakamichi *et al.* 2005) which is the major determinant of flowering in *Arabidopsis*. Thus the *PRR* gene presents a promising candidate for the *Ppd-H1* locus in barley. In common wheat, three homoeologous *Ppd* loci named *Ppd-A1*, *Ppd-B1* and *Ppd-D1* have been identified on the short arms of homoeologous group 2 chromosomes. Recently, Beales *et al.* (2007) have reported a photoperiodically-insensitive mutant of common wheat cv. Ciano 67 which carries a *Ppd-D1a* allele that has a 2 kb deletion upstream of the coding region of a *PRR* gene and is expressed under both LD and SD regimes. Therefore, it enhances flowering irrespective of the day-length, probably *via* a positive induction of *TaFT1* (*VRN-B3*), a major positive regulator of *VRN-1* expression, which is normally repressed under SD conditions.

Recently, Faure *et al.* (2007) have proposed that *HvFT3* gene, which belongs to the *HvFT* family (analogously as *HvFT1*, a candidate gene for *VRN3* locus) and which is involved in the regulation of flowering under SDs, can be a candidate gene for the *Ppd-H2* locus. It is also very probable that other HvFT TFs (the family of *HvFT* genes includes five members named *HvFT1* to *HvFT5* which encode proteins with a phosphatidyl-ethanolamine binding domain) are involved in photo-periodical regulation of the transition into flowering.

It has been demonstrated by many authors (Karsai *et al.* 2005, Dubcovsky *et al.* 2006) that the expression of *VRN* genes is affected by photoperiod. Dubcovsky *et al.* (2006) have found out that the expression of *VRN-2* gene is down-regulated by SDs. However, its down-regulation does not result in the up-regulation of *VRN-1* expression. Thus, the authors have postulated the existence of at least one other repressor of *VRN-1*. Kane *et al.* (2005) have described the *TaVRT-2* gene in wheat and its orthologue, *HvVRT-2* in barley, which is a MADS-box TF up-regulated by SDs and down-regulated by LDs in barley cv. Dicktoo. They have recently proven (Kane *et al.* 2007) that *TaVRT-2*, also a MADS-box TF, can bind to both the CArG box in the promoter of *VRN-1* and the *VRN-2* gene product. Thus, the model of *VRN-1* regulation becomes more complex and it seems evident that both vernalization and photoperiod affect the repression (de-repression) of *VRN-1*, and thus transition to flowering in many cultivars of wheat and barley. This model can also explain the long-known fact that facultative barley cultivars, which have a deletion in the *VRN2* locus, begin to flower significantly earlier under

Table 2. List of the major *Ppd* loci (their candidate genes, respectively) and photoperiodically regulated genes in barley which participate in the regulation of transition into flowering. In reference, *means discovery, **means mapping.

Locus	Gene	Location	Reference
Ppd-H1	*PRR*	2HS	Laurie *et al.* (1994) Karsai *et al.* (1997) Turner *et al.* (2005)
Ppd-H2	*HvFT3*	1HL	Laurie *et al.* (1995) Faure *et al.* (2007)
	HvVRT-2	7HS	Kane *et al.* (2005)* Szűcs *et al.* (2006)**
	HvCO1-9	1H,2H,5H,6H,7H	Griffiths *et al.* (2003)

LDs than under SDs (Table 2, Fig. 1). A very informative scheme of the relationships between individual vernalization- and photoperiodically-regulated loci (their candidate genes, respectively) in wheat and barley, compared with the analogous relationships in rice and *Arabidopsis*, has been published by Cockram *et al.* (2007b).

The induction of frost tolerance: the phenomenon of cold acclimation

Plants growing in regions where regular long periods of cold and/or frost during the year occur had adapted their life cycle to these unfavourable growth conditions. Annuals usually survive these periods as seeds, biennials and perennials survive them in the vegetative phase when their vegetative organs exhibit a sufficiently high level of FrT.

FrT is defined as the ability of plants to survive the impacts of frost (temperatures below zero) and is usually determined as LT_{50} values (the lethal temperature when 50 % of samples die). FrT is often inducible in many plant species including *Triticeae*; even very frost-tolerant plants are susceptible to frost when they are suddenly transferred from their optimum growth temperature to an environment with temperatures below zero. The distinction between frost-sensitive and frost-tolerant plants lies in the fact that frost-tolerant plants can induce a high level of FrT when exposed to a sufficiently long period of low, but above-zero temperatures, whereas frost-sensitive plants cannot. Non-acclimated rye, for instance, is killed by freezing at about -5 °C, but after a period of exposure to low nonfreezing temperatures the same rye can survive freezing down to about -30 °C (Thomashow 1999). Moreover, it usually takes quite a long time to reach the maximum FrT in highly frost-tolerant cultivars, *e.g.*, the plants of the highly frost-tolerant winter wheat Mironovskaya 808 reach the maximum FrT after a 4-week LT treatment at 3 °C (Prášil *et al.* 2004). This type of treatment, *i.e.*, the impact of low, above-zero temperatures on a plant, is called a process of cold acclimation (CA).

Moreover, the plant's ability to induce a sufficiently high level of FrT is dependent on its developmental stage. Species belonging to the tribe *Triticeae* are able to induce a high level of FrT only when they are in the vegetative phase of their individual development. The developmental transition into the reproductive phase, indicated in *Triticeae* by a double-ridge formation in the apical meristem, is usually accompanied by a significant decline in this ability (Fowler *et al.* 1996, 2001).

During CA, many physiological and biochemical changes in plant cells occur. They are aimed at the compensation of the loss of water in cell cytoplasm during LT treatment and at the avoidance of intracellular freezing. The osmotic potential of cell cytoplasm decreases due to the accumulation of osmotically active molecules of compatible solutes such as sugars (glucose, fructose, sucrose, mellibiose, raffinose, stachyose, verbascose), sugar alcohols (mannitol, sorbitol, pinitol), quaternary ammonium compounds (glycine betaine, alanine betaine), polyamines (spermine, spermidine, putrescine), and immino acid proline. In membranes, a fraction of unsaturated fatty acids increases in order to retain sufficient fluidity of the membranes and transmembranaceous protein complexes (Sakai and Larcher 1985, Guy 1990). Moreover, many cold-specific proteins are synthesized. These proteins can act either as enzymes – scavenging reactive oxygen species (ROS) which are produced in relatively high amounts under cold, synthesizing molecules of compatible solutes or unsaturated fatty acids, or they can have structural (protective) functions, *i.e.*, can act as chaperones to prevent other proteins and endomembranaceous structures from unfavourable structural changes. One important group of these structural proteins are COR/LEA (cold-regulated/late embryogenesis abundant) proteins which accumulate in both cell cytoplasm and nucleus under the conditions associated with cellular dehydration – environmental stress conditions (drought, enhanced salinity, enhanced evaporation, cold, frost) and the physiological conditions of embryo maturation and desiccation in the late stages of embryogenesis (hence the name LEA proteins).

As a consequence of the biochemical changes described above, the process of CA has increased demands on plant energetic metabolism. Therefore, the *Triticeae* are generally able to induce higher FrT levels under LD than under SD conditions when they are in the vegetative phase, since they can synthesize more assimilates under these conditions (Limin *et al.* 2007).

Cold-induced COR/LEA proteins, *CBF* genes and loci for frost-resistance (*Fr* loci)

COR/LEA proteins are a large group of important structural proteins that accumulate during cellular dehydration. They are usually highly hydrophilic and can protect other proteins and / or membranaceous structures against the loss of their hydration envelopes. The loss of water is associated with unfavourable structural and functional changes of the biomolecules. LEA proteins are usually divided into three major sub-groups based on their unique sequence characteristics (Ingram and Bartels 1996, Cattivelli *et al.* 2002). LEA I sub-group includes glycine-rich, highly hydrophilic proteins which contain one to four copies of a conserved 20 aa motif which consists of an N-terminal (GETWPGGTGGK) and a C-terminal (EGIDIDESKF) consensus sequences. LEA II

sub-group which is also called LEA D11 and whose members are named dehydrins includes all proteins with at least one copy of a lysine-rich sequence, the K-segment (consensus sequence EKKGIMDKIKEKLPG) which is the major antigen determinant of dehydrins (Close *et al.* 1993) and which can form a class A2 amphipathic α-helix under conditions of reduced hydration (Close 1996, 1997). LEA III sub-group is characterised by the presence of a tandem repeat of an 11 aa sequence (consensus ΦΦE/QxΦKE/QKΦxE/D/Q where Φ represents a hydrophobic aa), which can form an amphipathic α-helix (Dure 1993).

In wheat and barley, the cold-induced *Lea* genes include many *Lea II* genes – dehydrins; in barley, the induction by cold has been described for *Dhn5* (K_n type) and *Dhn8* (acidic SK_3 type) (Choi *et al.* 1999); in common wheat, the induction by cold has been described for *Wcs120* gene family including *Wcs200*, *Wcs180*, *Wcs66*, *Wcs120*, *Wcor825* and *Wcor726* genes (all K_n type; for review, see Sarhan *et al.* 1997), acidic SK_3-type *Wcor410* gene family including *Wcor410a*, *Wcor410b* and *Wcor410c* genes (Danyluk *et al.* 1994; 1998); small K_n-type dehydrin *Wdhn13* (Ohno *et al.* 2003), *etc.* From *Lea III* sub-group, induction by cold has been described for chloroplast-located *Wcs19*, *Wcor14a,b* and *Wcor15* in wheat and *Cor14b* in barley and for many other *Cor* and *Rab* genes. Some of the cold-induced *Cor*/*Lea* genes can be regarded as markers of FrT, *i.e.*, their amounts accumulated under cold correspond quantitatively with the level of FrT in different cultivars of wheat and barley (*e.g.*, the WCS120 proteins in wheat – Houde *et al.* 1992b, Vítámvás *et al.* 2007, DHN5 protein in barley – Kosová *et al.* 2008, *Cor14b* in barley and wheat – Crosatti *et al.* 1995, Vágújfalvi *et al.* 2000; for review on the roles of dehydrins upon cold, see Kosová *et al.* 2007). A positive effect of the expression of some wheat *Cor/Lea* genes on the enhancement of FrT has also been proven by studies using transgenic techniques (*e.g.*, NDong *et al.* 2002, Shimamura *et al.* 2006).

The expression of *Cor*/*Lea* genes is regulated by several regulatory pathways which can be basically divided into ABA-dependent and ABA-independent (Table 3). The *Cor*/*Lea* genes whose expression is predominantly regulated by ABA contain several ABRE regulatory elements in their promoter regions which serve as a binding site for bZIP TFs. The ABRE elements possess two fragments: TACGTCC (the G-box) and GGCCGCG (GC-motif) (Thomashow 1999, Allagulova *et al.* 2003, Yamaguchi-Shinozaki and Shinozaki 2005).

One of the most important ABA-independent regulatory pathways is the CBF pathway. In *Arabidopsis*, four *CBF* TFs (*CBF1* - *CBF4*) have been identified; three of them (*CBF1*/*DREB1B*, *CBF2*/*DREB1C* and *CBF3*/*DREB1A*) are cold-induced and tandemly arranged on chromosome 4 while *CBF4*/*DREB1D* is drought-induced. The CBF TFs bind to CRT/DRE/LTRE regulatory elements in promoter regions of their effector genes (*e.g.*, many *Cor*/*Lea* genes). The CRT/DRE/LTRE elements contain a characteristic sequence GCCGAC which serves as a binding site for the AP2 domain of the *CBF* TF (Yamaguchi-Shinozaki and Shinozaki 2005). The expression of *CBF* TFs in *Arabidopsis* is controlled partly by *ICE1* TF (inducer of *CBF* expression 1; TF with bHLH DNA-binding domain) which binds to MYC elements in the promoter regions of the *CBF* genes (Chinnusamy *et al.* 2003); by *ICE1*, the expression of *CBF3* gene is predominantly regulated while *CBF1* and *CBF2* genes are influenced only very slightly. In addition to *ICE1*, it has been shown by Vogel *et al.* (2005) that *ZAT12* regulates the expression of *CBF* genes in *A. thaliana*. Recently, one *AtICE1* homologue and two *AtZAT12* homologues have been identified in barley by Skinner *et al.* (2006) by *BLAST* search of EST sequences. Zarka *et al.* (2003) have found threshold induction temperature for *CBF* genes in *A. thaliana* around 14 °C. Jaglo-Ottosen *et al.* (1998) have clearly shown the relationship between the expression of *CBF* genes and *Cor* genes in *A. thaliana* – the overexpression of *CBF1* led to enhanced expression of four *Cor* genes. In common wheat, a positive relationship between the activity of *CBF* genes and the expression of *Cor*/*Lea* genes has been confirmed by Kobayashi *et al.* (2005), Kume *et al.* (2005), and others.

The majority of *CBF* genes in *Triticeae* has been mapped to the long arm of homoeologous group 5 chromosomes at the *Fr-2* locus (Choi *et al.* 2002, Francia *et al.* 2004), one of the two major QTLs for FrT which is also one of the two major QTLs regulating the expression of *Cor14b* gene (Vágújfalvi *et al.* 2000, 2003). It has also become evident that the *CBF* genes in *Triticeae* are more numerous [barley – 20 *CBFs* (Skinner *et al.* 2005), *T. monococcum* – 13 *CBFs* (Miller *et al.* 2006), *T. aestivum* – up to 25 *CBFs* proposed (Badawi *et al.* 2007)] and diverse than in *Arabidopsis*. In barley and in *T. monococcum*, the *CBFs* have been divided into three distinct phylogenetic sub-groups (Skinner *et al.* 2005, Miller *et al.* 2006). In *T. aestivum*, the *CBFs* have been divided into ten sub-groups (Badawi *et al.* 2007), six of them have been characterised as *Pooideae*-specific. It is also interesting that in both wheat and barley, the members of the sub-groups, which are most closely sequentionally related to the *Arabidopsis CBF1* – *CBF3* genes, are located in chromosomal regions other than the *Fr-2* loci.

This large diversity of *CBFs* in *Triticeae* may be a consequence of their adaptation to temperate climate habitats. Expression studies carried out by Badawi *et al.* (2007) showed that five of the *Pooideae*-specific sub-groups display higher constitutive and CA-inducible expression levels in the winter wheat cultivar Norstar when compared to the spring wheat cultivar Manitou. The higher constitutive and inducible expression levels probably present an inherited trait of the winter cultivars, which may form the basis of the higher FrT capacity of the winter cultivars when compared to the spring ones. The quantitative differences in *CBF* expression between differently frost-tolerant cultivars have also been

observed by other researchers in einkorn wheat (Vágújfalvi *et al.* 2005, Miller *et al.* 2006) and in barley (Stockinger *et al.* 2007). The last authors mentioned above have also confirmed differences in constitutive expression of some *CBFs* at the *Fr-2* locus. They have also found out that the quantitative expression of some barley *CBFs* is dependent on photoperiod. However, other studies determining the expression levels of individual *CBFs* under cold and investigating the redundancy between them will be needed to characterise the contribution of individual CBFs to the expression of *Cor* genes and induction of FrT.

Other studies have dealt with the affinity of CBFs to various CRT motifs occurring in the promoters of *Cor* genes. Xue (2002) demonstrated the interaction of HvCBF1 with the (G/a)(C/t)CGAC sequence present in the promoters of *Cor* genes. The activation of the wheat *Cor* genes *Wdhn13* and *Wrab17* by the wheat WCBF2 was demonstrated by Takumi *et al.* (2008) in transgenic tobacco. Skinner *et al.* (2005) have confirmed a great variability in the binding affinity of different CBFs to individual CRT motifs which depends on the sequence motifs flanking the CRT core sequence CCGAC. In addition, Xue (2003) has shown that the binding affinity of the AP2 domains of the CBFs is also temperature-dependent; the author has found out that a temperature decline leads to the enhanced affinity of HvCBF2 to the GCCGAC core motif of the CRT/DRE/LTRE elements. Thus, it can be proposed that a temperature-dependent binding affinity of CBFs can present another level of regulation of *Cor* gene expression.

Apart from the *Fr-2* locus, the major frost-resistance loci named *Fr-1* have been mapped to the long arms of homoeologous group 5 chromosomes in the *Triticeae* genomes far from the *Fr-2* loci, but in a tight linkage with the *VRN-1* loci. Thus, in barley, the *Fr-H1* locus is tightly linked to the *VRN-H1* locus, and in hexaploid wheat, the *Fr-A1* locus is linked to the *VRN-A1* locus (interval of 2 cM – Galiba *et al.* 1995), the *Fr-B1* locus is linked to the *VRN-B1* locus (interval of 40 cM – Tóth *et al.* 2003), and the *Fr-D1* locus is linked to the *VRN-D1* locus (interval of 10 cM – Snape *et al.* 1997). The two *Fr* loci on the long arm of chromosome 5 have also been described by Vágújfalvi *et al.* (2000) as QTLs affecting the expression of *Cor14b* gene in wheat. The expression study carried out by Kobayashi *et al.* (2005) in *T. aestivum* suggested that the *Fr-1* loci can control *Wcbf2* gene expression and thus the up-regulation of the downstream *Cor*/*Lea* gene expression at least partly through the CBF pathway.

In wheat, it is suggested that the spring-type *Vrn-A1* allele is associated with a spring-type *Fr-A1* allele and conversely, the winter-type *vrn-A1* allele is tightly linked to winter-type *Fr-A1* allele. In the B and D genomes, the situation is proposed to be analogous. As *Vrn-A1* is the major *VRN-1* gene in common wheat, it is also proposed that the *Fr-A1* gene has the major effect on the expression of cold-induced genes when compared with the *Fr-B1* and *Fr-D1* genes. Kobayashi *et al.* (2005) used a set of near isogenic lines (NILs) of common wheat and found out that the *Vrn-B1* NIL showed a higher FrT than the *Vrn-A1* NIL because the *Vrn-B1* NIL probably possessed a dominant winter-type *Fr-A1* allele while the *Vrn-A1* NIL possessed a spring-type *Fr-A1*. Similarly, Sutka *et al.* (1999) showed that the lines possessing a functional *Fr-A1* locus showed a 13 % higher survival rate in frost tolerance tests compared to the lines lacking it. Some authors (Miller *et al.* 2006) have even proposed that in genome A, the *Fr-A1* locus is so tightly linked to the *Vrn-A1* locus that they may be identical and that the *Vrn-A1* gene product could regulate the expression of *CBF* genes. However, it has been shown by Ishibashi *et al.* (2007) that wheat cultivars possessing either a dominant *Vrn-D1* allele or a recessive *vrn-D1* allele can exhibit no significant differences in FrT. Therefore, the authors have suggested that in the D genome, the *Vrn-D1* and *Fr-D1* loci are not tightly linked together, and as a consequence, a similar level of acquired FrT can be found in the winter and spring wheat cultivars differing only in the allelic constitution of the *VRN-D1*. Recently, it has been found out that the *Fr* locus described originally as *Fr-B1* locus by Tóth *et al.* (2003) is orthologous to the *Fr-A2* locus in wheat; thus, its name has been corrected to *Fr-B2* (McIntosh *et al.* 2004). Analogously to wheat, the *Fr-H1* locus in barley is tightly linked to the *VRN-H1* locus and it has been shown by Francia *et al.* (2004) that the QTL for FrT on 5 HL cannot be clearly separated from the QTL for vernalization requirement in the Nure (winter) × Tremois (spring) barley mapping population. The candidate genes for the *Fr-1* locus in *Triticeae*, the major locus controlling the induction of FrT, have not been identified yet.

Studies dealing with the relationships between the activity of the VRN-1 gene products and *Cor*/*Lea* gene expression have clearly shown that the induction of the formation of flower meristem and the induction of *VRN-1* gene expression lead to the decline in *Cor*/*Lea* gene expression (Fowler *et al.* 2001, Danyluk *et al.* 2003, Kane *et al.* 2005, Kobayashi *et al.* 2005, Kume *et al.* 2005). Recently, Stockinger *et al.* (2007) have reported that the *VRN-H1*/*Fr-H1* locus affects the expression of multiple *CBF* genes at the *Fr-H2* locus in the Nure × Tremois barley mapping population. Prášil *et al.* (2005) have also shown that different *vrn-1* alleles affect the ability to maintain a sufficient level of FrT during the conditions of CA in winter wheat. However, the interactions between the activity of *VRN-1* and *Cor*/*Lea* gene expression and the development of FrT have not been precisely elucidated yet.

A scheme describing the possible regulatory relationships between the vernalization loci (the candidate genes), the photoperiod loci (the candidate genes) and the frost-resistance loci (the candidate genes) is given in Fig 1.

Table 3. The list of major cold-induced *Cor/Lea* genes in wheat and barley. Genes and corresponding proteins are characterised by their accession numbers from NCBI (state in March 2008), pI and M_r of the proteins were calculated by a calculation tool in *ExPASy* (Swiss protein database). The values of Mr in brackets were determined by SDS-PAGE.

Gene (accession number)	Protein (accession number)	Group LEA	Number of aa	pI	M_r [kDa]	Reference
Triticum aestivum						
Wcs200	WCS200 (AAB31285)	II		6.5	(200)	Quellet *et al.* (1993)
Wcs180	WCS180	II				Houde *et al.* (1995)
Wcs66 (L27516)	WCS66/CS66 (AAA21819)	II	469	6.74	46.8	Chauvin *et al.* (1994)
Wcs120 (M93342)	WCS120/CS120 (AAA34261)	II	390	7.02	39 (50)	Houde *et al.* (1992a)
Wcs40	WCS40	II		7.3	(40)	Houde *et al.* (1995)
Wcs726/Wcor726 (U73213)	WCS726/WCOR726 (AAB18204)	II	124	7.04	12.7	Danyluk and Sarhan (1996 – NCBI)
Wcs80/Wcor80 (U73212)	WCS80/WCOR80 (AAB18203)	II	93	8.05	9.6	Danyluk and Sarhan (1996 – NCBI)
Cor39 (AF058794)	COR39 (AAC14297)	II	391	6.92	39 (50)	Guo *et al.* (1992)
Wdhn13 (AB076807)	WDHN13 (BAC01112)	II	124	8.01	12.8	Ohno *et al.* (2003)
Wcor410a (L29152)	WCOR410a (AAA20189)	II	262	5.19	28	Danyluk *et al.* (1994)
Wcor410b (U73210)	WCOR410b (AAB18201)	II	268	5.25	28.8	Danyluk and Sarhan (1996 – NCBI)
Wcor410c (U73211)	WCOR410c (AAB18202)	II	259	5.2	27.9	Danyluk and Sarhan (1996 – NCBI)
Wcor825 (U73215)	WCOR825 (AAB18206)	II	73	8.08	8.09	Danyluk and Sarhan (1996 – NCBI)
Wcor14a (AF207545)	WCOR14a (AAF17098)	III	140	4.86	13.5	Tsvetanov *et al.* (2000)
Wcor14b (AF207546)	WCOR14b (AAF17099)	III	137	9.1	13.6	Tsvetanov *et al.* (2000)
Wcor15b(AB095006)	WCOR15 (BAC56935)	III	147	5.12	14.7	Takumi *et al.* (2003)
Wcor615 (U73217)	WCOR615 (AAB18208)	III	175	4.92	17.8	Danyluk and Sarhan (1996 – NCBI)
Wlt10 (AF271260)	WLT10 (AAF75555)	III	101	6.52	9.9	Tsvetanov *et al.* (2000)
Wcs19 (L13437)	WCS19 (AAA16282)	III	147	5.59	14.6	Chauvin *et al.* (1993)
Wrab15 (AB115913)	WRAB15 (BAC80265)	III	130	7.9	15	Kobayashi *et al.* (2004)
Wrab17 (AF255053)	WRAB17 (AAF68628)	III	166	4.85	17.2	Tsuda *et al.* (2000)
Wrab18 (AB115914)	WRAB18 (BAC80266)	III	169	5.95	17.5	Kobayashi *et al.* (2004)
Wrab19 (AF255052)	WRAB19 (AAF68627)	III	179	8.63	18.3	Tsuda *et al.* (2000)
Hordeum vulgare						
Dhn5 (AF181455)	DHN5 (AAF01695)	II	575	6.65	58.5 (86)	Close *et al.* (1995)
Dhn8 (AF181458)	DHN8 (AAF01696)	II	255	5.21	27.7	Choi *et al.* (1999)
Cor14b (AJ512944)	COR14b (CAD55692)	III	142	4.84	13.9	Dal Bosco *et al.* (2003)

Future perspectives

In recent years, significant progress in the research dealing with developmental and environmental regulation of FrT in *Triticeae* has been made. However, many crucial questions still remain unanswered. Now, we would like to discuss the two of them in detail.

1) It becomes evident that the VRN-1 gene product is the main regulator of the developmental transition to the less frost-tolerant reproductive phase. This hypothesis has recently been confirmed by Shitsukawa *et al.* (2007) who have prepared and further characterised a mutant *maintained vegetative phase* (*mvp*) of *T. monococcum* which does not flower as a consequence of a deletion of the promoter and coding regions of *VRN-1* gene. In contrast, Adam *et al.* (2007) have observed enhanced transition to flowering in transgenic *Arabidopsis* plants overexpressing the *VRN-1* gene from common wheat. Therefore, the regulation of the *VRN-1* gene expression seems to present a powerful tool how to affect the plant's potential to induce a sufficiently high level of FrT under the conditions of CA and to maintain it for a sufficiently long period of time. Detailed regulation of *VRN-1* gene expression is still unknown. However, it becomes obvious that the VRN-2 gene product is not the only repressor of the *VRN-1* gene. Kane *et al.* (2005) have described *VRT-2* gene, a MADS-box gene distinct structurally from the *VRN-2* gene (a *ZCCT* gene) as another repressor of the *VRN-1* gene, whose expression is maintained by SDs in barley (in contrast to the *VRN-2*

gene whose expression is down-regulated by SDs). It also becomes evident that the *VRN-1* gene is under positive regulation of a LD- responsive pathway mediated by VRN-3 gene product.

Another mechanism, which regulates the transition to flowering in *Arabidopsis*, but has not experimentally been studied in *Triticeae* yet, presents the epigenetic regulation of gene activity *via* chromatin modification. It is well known from *Arabidopsis* that the silencing of the major flowering repressor *FLC* is mediated via histone methylation. In wheat and barley, no experimental evidence of chromatin modification at the vernalization loci has been found yet, but it can be expected that the changes in pattern of chromatin modification may play an important role in the changes of gene expression associated with the effect of LD photoperiod after the fulfillment of vernalization requirement. Trevaskis *et al.* (2007) have hypothesized that the activity of *VRN-1* gene can be regulated via chromatin modification and that the 'vernalization critical region' in the first intron of this gene might be a target of protein complexes that regulate chromatin modification. According to this model, the expression of the recessive *vrn-1* alleles would be repressed via chromatin methylation and LD pathway would activate protein complexes which would change pattern of chromatin modification after the fulfillment of vernalization requirement (down-regulation of *VRN-2* gene), thus leading to the activation of *VRN-1* gene. Three homologues of *A. thaliana VIL* (*Vernalization-Insensitive 3 Like*) genes, which regulate the epigenetic silencing of *FLC* after vernalization in *Arabidopsis*, have been recently found in *T. monococcum* by Fu *et al.* (2007). Experimental evidence which would confirm or reject this hypothesis is still lacking.

However, a creation and testing of a complex model of the expression of the *VRN-1* gene presents a great challenge for scientists studying the regulation of the developmental transition in *Triticeae*.

2) It has been confirmed by many researchers that the activity of the *VRN-1/Fr-1* locus is crucial for the *Triticeae'* ability to develop a sufficient level of FrT upon CA. However, the precise role of the *VRN-1/Fr-1* locus in this process remains to be elucidated. First, until now, the nature of the *VRN-1/Fr-1* locus itself is still not clear, *i.e.*, it is not clear whether the FrT QTL mapped to this locus is the effect of some yet unidentified gene or simply a pleiotropic effect of the *VRN-1* gene itself. Therefore, two possible hypotheses could be formulated:

a) The *VRN-1/Fr-1* locus contains two different genes – the *VRN-1* gene affecting the vernalization response and another, yet uncharacterised gene responsible for the induction of FrT and regulation of the *Fr-2* locus. A possible scenario could be the following: both genes are tightly linked together, *i.e.*, there are no recombination events between them. Thus, winter genotypes, which have only recessive *vrn-1* alleles, also have only winter-type *Fr-1* alleles, which strongly activate the *Fr-2* located genes (the *CBF* genes). In contrast, spring genotypes, which have at least one dominant *Vrn-1* allele, possess at least one spring-type *Fr-1* allele, which can activate the *Fr-2* genes only weakly. Therefore, the winter cultivars in the vegetative phase can induce a higher FrT than the spring ones. During developmental transition into the reproductive phase, the *VRN-1* gene expression is induced and the *VRN-1* gene product then represses the activity of *Fr-1*.

b) According to the latter scenario, the FrT QTL is simply one effect of the activity of the *VRN-1* gene. It can be proposed that the *VRN-1* gene product acts, in coordination with some other TFs, as a repressor of the *CBF* genes at the *Fr-2* locus. Winter cultivars, where the *vrn-1* allele is repressed under LT and SD conditions, can thus induce high activity of *Fr-2* genes via signalling pathways that respond directly to LT (*e.g.*, *via ICE-1* or *ZAT12* homologues). Since the activity of the *CBFs* is not repressed in these cultivars, they are able to induce a high level of FrT prior to the fulfillment of their vernalization requirement. In contrast, spring cultivars cannot repress the activity of *Vrn-1*, thus the *Vrn-1* acts as a repressor of *CBFs* at the *Fr-2* locus and the activity of *CBFs* at this locus under CA is a result of a positive regulation by some signalling pathways (maybe via *ICE-1* or *ZAT12* homologues) and a negative regulation by *Vrn-1*. Thus, the resulting activity of the *CBFs* at *Fr-2* in spring genotypes upon CA is weaker compared to the winter genotypes prior to the fulfillment of vernalization requirement; therefore, the spring genotypes can induce an enhanced FrT upon CA only transiently and at lower levels than the winter ones. A transient activity of CA-induced genes in a spring wheat cultivar compared to a winter one under cold has recently been found by Monroy *et al.* (2007) on a *T. aestivum*-specific microarray.

The characterization of the *VRN-1/Fr-1* locus thus remains a task for scientists who study the genetic basis of the impact of individual development on the ability to induce FrT in *Triticeae*. Recently, Stockinger *et al.* (2007) have confirmed the effect of the *VRN-1/Fr-1* region on the regulation of some *Fr-2* located *CBF* genes in the Nure × Tremois barley mapping population. These authors have also hypothesized that the QTL for FrT may be a pleiotropic effect of the *VRN-1* gene and that an allelic variation in *VRN-1* may exist which can cause that some *VRN-1* alleles have a strong effect on flowering, but no effect on *CBF* gene expression and development of FrT. The allelic variation in *VRN-1* could explain the QTL for FrT in the reproductive stage mapped to the *VRN-1/Fr-1* region by Reinheimer *et al.* (2004). However, other experiments need to be conducted to investigate the role of *VRN-1/Fr-1* region in *CBF* gene expression and development of FrT in *Triticeae*. A detailed comprehension to this regulatory pathway will certainly contribute to our better understanding of the mechanisms which regulate the development of FrT in *Triticeae* with respect to the plant individual development. Furthermore, the results of this research may be useful for the breeders in order to design new varieties of wheat and barley with known physiological characteristics which will be able to better respond to

changing environments.

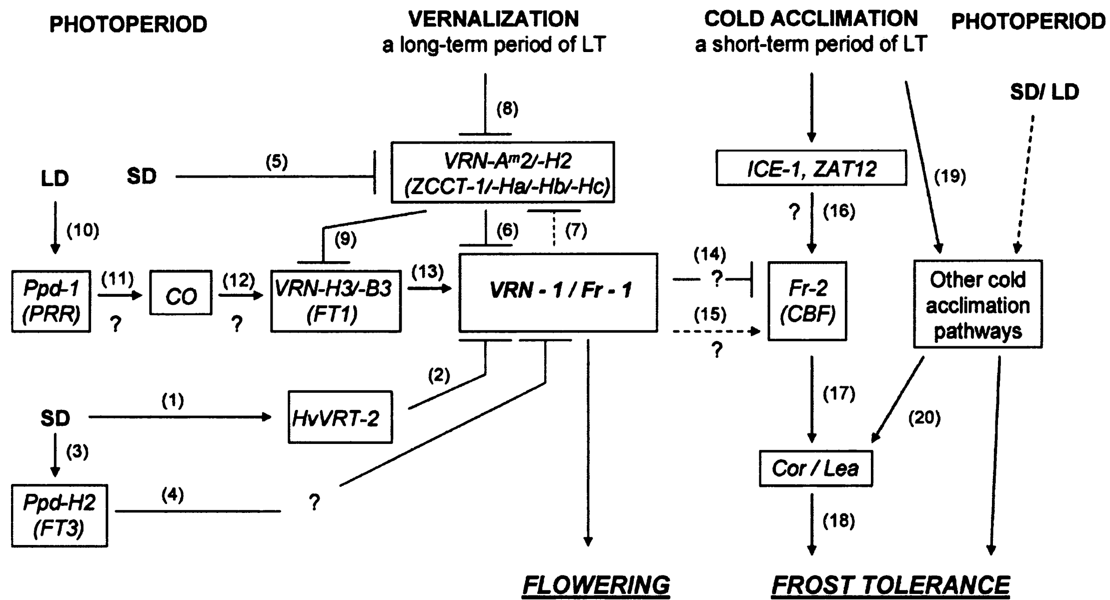

Fig. 1. A descriptive scheme of the relationships between vernalization- and photoperiodically-regulated loci (their candidate genes, respectively) which regulate the transition to flowering and loci which regulate the development of frost tolerance (FrT) upon cold acclimation (CA). (1) Kane *et al.* (2005); (2) Kane *et al.* (2007); (3) Laurie *et al.* (1995); (4) Faure *et al.* (2007); (5) Yan *et al.* (2004b); (6) when *vrn-1* is recessive and *Vrn-2* is dominant; Von Zitzewitz *et al.* (2005); Szűcs *et al.* (2007); (7) possibly when *Vrn-1* is dominant or when *vrn-1* is recessive under prolonged LD treatment; Loukoianov *et al.* (2005); Trevaskis *et al.* (2006); (8) Dubcovsky *et al.* (2006); Trevaskis *et al.* (2006); (9) Yan *et al.* (2006); (10) Laurie *et al.* (1994); (11) Turner *et al.* (2005); Beales *et al.* (2007); (12) Griffiths *et al.* (2003); Turner *et al.* (2005); (13) Yan *et al.* (2006); (14) Danyluk *et al.* (2003); Kane *et al.* (2005); Kobayashi *et al.* (2005); Stockinger *et al.* (2007); (15) Vágújfalvi *et al.* (2000); Kobayashi *et al.* (2005); Stockinger *et al.* (2007); (16) Skinner *et al.* (2006); (17) Vágújfalvi *et al.* (2000); Choi *et al.* (2002); Kume *et al.* (2005); Skinner *et al.* (2005); Miller *et al.* (2006); (18) Houde *et al.* (1992b); Crosatti *et al.* (1995); Vágújfalvi *et al.* (2000); Vítámvás *et al.* (2007); Kosová *et al.* (2008); (19) Thomashow (1999); (20) Choi *et al.* (1999); Thomashow (1999). Question marks indicate uncertain or unknown components of the regulatory pathways, dashed lines indicate uncertain regulatory relationships.

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REVIEW

The role of dehydrins in plant response to cold

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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 unfavourable 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 unfavourable conditions. It is demonstrated that the induction of dehydrin expression and their accumulation is an important part of this process in many dicotyledons (both herbaceous and woody species), and also in winter cultivars of cereals, especially wheat and barley. Some mechanisms which are proposed to be involved in regulation of dehydrin expression are discussed, *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. Finally, we outline some new approaches emerging for the solution of the complex mechanisms involved in plant cold-acclimation, especially the methods of functional genomics that enable to observe simultaneously changes in the activity of many genes and proteins in a single sample.

Additional key words: abscisic acid, cereals, cold-acclimation, dicotyledons, frost resistance, K-segment, LEA D-11 proteins, low temperature stress.

Introduction

Dehydrins, also known as LEA D-11 or LEA II (late embryogenesis abundant) proteins, are proteins whose expression is induced by various environmental factors, which cause dehydration of the cells. Among these factors, cold, frost, heat, drought, salinity, and enhanced evaporation are the most notable (e.g., Wisniewski *et al.* 1996, Buchanan *et al.* 2005, Rampino *et al.* 2006, Wahid and Close 2007). Expression of many dehydrins is also induced by increased abscisic acid (ABA) content. The classification of LEA proteins originates from sequence homologies of late embryo-genesis abundant proteins from cotton to LEA proteins from other plant species. Currently, LEA proteins are divided into 5 groups: LEA D19 (group I), LEA D11 (group II, also termed dehydrins), LEA D7 (group III), LEA D113 (group IV), and LEA D95 (group V) (Ingram and Bartels 1996). Currently, dehydrins are considered all the proteins which have at least one copy of the lysine-rich amino acid

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Abbreviations: ABA - abscisic acid; ABRE - ABA-responsive element; bZIP - basic-domain leucine zipper; CaMV - cauliflower mosaic virus; CAT - catalase; CBF - C-repeat-binding factor; *Cor* - cold-regulated; CRT - C-repeat; *Dhn* - dehydrin; DRE - dehydration-responsive element; ELIPs - early light-inducible proteins; *Erd* - early response to drought; EST - expressed sequence tag; *Fr* gene - frost resistance gene; FT - frost tolerance; GUS - β-glucuronidase; LEA - late embryogenesis abundant; LD - long day; LDH - lactate dehydrogenase; LT - low temperature; LT_{50} - lethal temperature when 50 % samples die; *Lti* - low temperature-induced; LTRE - low temperature-responsive element; M_r - relative molecular mass; NLS - nuclear localisation sequence; PD_{50} - 50 % protein denaturation; pI - isoelectric point; Ppd - photoperiod; QTL - quantitative trait loci; *Rab* - response to ABA; RT-PCR - reverse transcriptase polymerase chain reaction; SD - short day; SDS-PAGE - sodium dodecyl sulphate polyacrylamide gel electrophoresis; UV CD - ultra-violet circular dichroism; Vrn - vernalization; *Wcor* - wheat cold-regulated; *Wcs* - wheat cold-specific; *Wdhn* - wheat dehydrin; WT - wild type; 2DE - two dimensional electrophoresis; 2D-DIGE - two dimensional difference gel electrophoresis.

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sequence, the K-segment, in their molecule. Hence, a shift in the definition of dehydrins in the past two decades can be noted: from a function-based definition (dehydrins as dehydration-induced proteins) to a sequence-based definition (dehydrins as proteins with K-segment) (Close 1997).

The K-segment is usually located near the carboxy-terminus and has a consensus sequence EKKGIMDKIKEKLPG (Close 1996, Campbell and Close 1997, Close 1997). A polyclonal antibody was raised against the dehydrin K-segment which can be used for detection of dehydrins in a wide range of angiosperms and gymnosperms (Close *et al.* 1993). It is proposed that the K-segment forms an amphipathic class A2 α-helix, *i.e.*, the hydrophobic amino acid residues are located on one side of the helix and the residues of the polar amino acids on the other (Close 1996, 1997, Velten and Oliver 2001). It has been proposed by Ingram and Bartels (1996) that the repeating K-segments of a α-helical structure may form intramolecular bundles which probably enhance their amphipathic character. The dehydrins may function as emulsifiers or chaperones in the cells (Close 1997, Allagulova *et al.* 2003), *i.e.*, that they can interact with cell endomembranaceous systems or partially unfolded proteins *via* the hydrophobic side of the K-segment and thus protect them against unfavourable changes during dehydration (Egerton-Warburton *et al.* 1997, Danyluk *et al.* 1998, Koag *et al.* 2003). It has been proposed by Israelachvili and Wennerstrom (1996) that under a well-hydrated state, individual macromolecules like dehydrins and phospholipids in the membranes are surrounded by highly ordered water molecules. Therefore, they cannot interact with each other. During dehydration, the "water envelope" disrupts and the macromolecules then can come into mutual interaction.

Apart from the K-segment, which occurs in 1 to 11 copies in dehydrin molecules, dehydrins may contain other conserved regions – the Y-segment. It is located near the amino terminus and the S-segment, which contains multiple serine residues, whose functions can be modified via phosphorylation (Close 1996, 1997). It has been proposed that the phosphorylated serine tract can act as a nuclear localisation signal (NLS) and can lead to the accumulation of dehydrins in the nucleus (Close 1996, 1997). The accumulation of dehydrins in the nucleus was observed by Egerton-Warburton *et al.* (1997) in embryo tissue of *Zea mays* using immunogold labelling after application of ABA. Apart from the Y-, S- and K-segments, dehydrins are characterised by the presence of less conserved regions which are often rich in glycine and polar amino acids, the Φ-segments. It has been suggested (Ingram and Bartels 1996, Danyluk *et al.* 1998) that the Φ-segments have a structure of random coil which enables them to bind substantial amounts of water due to interactions of dipolar peptide bonds with water molecules. These regions contribute significantly to the typical characteristics of dehydrins, which are: *1*) high hydrophilicity, *2*) solubility in aqueous solutions upon boiling, and *3*) high affinity to detergents such as sodium dodecyl sulphate (SDS). In consequence, the apparent molecular mass (M_r) of the dehydrins on electrophoretic gels appears significantly higher than the actual M_r of these proteins calculated from their amino acid sequence (Close 1997, Ismail *et al.* 1999a). Some dehydrins have fairly polar and negatively charged amino acids instead of glycine in their primary sequence and can be characterised by relatively low pI values. These dehydrins such as WCOR410 in wheat or DHN8 in barley may represent a distinct acidic sub-group among dehydrins (Danyluk *et al.* 1994). Dehydrins nearly lack cysteine and tryptophan (Close 1997).

In aqueous solutions, it has been postulated and also experimentally proven by Ismail *et al.* (1999a) and Hara *et al.* (2001) using a technique of far-ultraviolet circular dichroism (far-UV CD) that dehydrins have a random coil secondary structure. However, their K-segments adopt an amphipathic α-helical structure in the presence of detergents such as SDS. These observations can lead to the hypothesis that dehydrins change their biochemical properties in dependence on whether or not they are in contact with any membranaceous structures or not.

Generally, according to the presence of the Y-, S- and K-segments, dehydrins can be divided into some biochemically different sub-groups: the Y_nSK_2 dehydrins, the K_n dehydrins, the K_nS dehydrins, the SK_n dehydrins, and the Y_2K_n dehydrins (Close 1996, 1997, Campbell and Close 1997, Svensson *et al.* 2002, Allagulova *et al.* 2003).

Cold stress and its physiological impacts on plants

Dehydration during cold stress often occurs as a result of an imbalance between reduced root water uptake and unchanged leaf transpiration (Sakai and Larcher 1987). The formation of ice crystals in extracellular spaces during frost stress also diminishes the portion of liquid water in the cells and causes dehydration due to the decrease in water potential outside the cells. The severity of cold and frost stress plays a pivotal role in the winter survival of some groups of plants including winter cereals and some economically important woody species such as birch, peach or poplar.

The cold stress (also known as chilling) includes low, above-zero temperatures ranging from 0 to 12 or 15 °C. These temperatures can cause severe damage to plants of tropical and sub-tropical origin, *i.e.*, chilling-sensitive plants such as *Zea mays*, *Glycine max*, *Lycopersicon* sp., *Cucumis sativus* or *Gossypium hirsutum*. In contrast, these temperatures induce important biochemical and physiological changes in freezing-tolerant plants such as winter cultivars of *Secale cereale*, *Triticum aestivum* or *Hordeum vulgare* which help them to survive sub-zero temperatures, *i.e.*, freezing.

The cold stress which usually precedes freezing in natural environments induces important changes in the composition of cytoplasm and biomembranes. It induces osmotic adjustment of the cytoplasm, *i.e.*, accumulation of compatible solutes – low-molecular mass, highly soluble compounds such as monosaccharides, oligosaccharides (sucrose, raffinose and raffinose-derived oligosaccharides: stachyose, verbascose, and disaccharide mellobiose), sugar alcohols (sorbitol, mannitol, pinitol), polyamines (spermine, spermidine, putrescine), quaternary ammonium compounds (glycinebetaine, alaninebetaine) or proline. They decrease the water potential of the cell compartments and prevent the formation of intracellular ice crystals by decreasing threshold freezing temperature of the cytoplasm (Sakai and Larcher 1987). Intracellular ice crystals cause irreversible damage of cell structures and they are usually lethal for the cell (Guy 1990). The exceptions are those cells which can deep-supercool, *i.e.*, their cytoplasm can acquire the character of a supersaturated glass-like solid phase. Glass-like matter prevents the intracellular compartments from mechanical collapse and enables the cell to avoid the formation of intracellular ice crystals. It seems very likely that the cytosolic composition of soluble sugars plays a crucial role in the formation of the glass-like state (Ingram and Bartels 1996). The membranes, especially the plasmalemma, are the primary site of the freezing injuries (Sakai and Larcher 1987, Thomashow 1999) undergoing several changes in their biophysical properties associated with liquid crystalline to gel and lamellar-to-hexagonal II phase transitions. When the plasmalemma is damaged, the leakage of soluble cytosolic compounds leads to the death of the cell. Therefore, the undamaged plasmalemma is crucial for the survival of the whole cell. To save undamaged membranes, important biochemical changes in their composition occur under cold conditions. In membranes, the content of sterols decreases and the fraction of phospholipids with unsaturated fatty acids increases during cold as a result of an adaptation which should help to maintain membrane fluidity necessary for lateral movements of important transmembrane complexes. Frost then leads to formation of ice crystals in extracellular spaces. Ice crystals lead to the drop in water potential outside the cell and show a strong tendency to draw water from the cytoplasm, but the decreased water potential of the cytoplasm often manages to prevent high water loss. Apart from membrane damage caused by severe dehydration due to the decreased water potential of the extracellular spaces it has been shown that some additional factors such as reactive oxygen species and mechanical adhesions of ice crystals can significantly contribute to the rupture of cellular membranes (Thomashow 1999).

Cold acclimation also leads to the accumulation of soluble proteins in cell cytoplasm in various plant tissues including dry and imbibed seeds. This phenomenon was described by many authors, *e.g.*, by Kumar and Bhatla (2006) in the seeds of chilling-sensitive sunflower (*Helianthus annuus*). One important component of plant mechanisms which protect both the cytoplasm and the membranes against excess water loss is the accumulation of dehydrins as proteins with the special features which were mentioned above. Due to their unique composition which enables them to bind substantial amounts of water, dehydrins can also be considered compatible solutes (Ingram and Bartels 1996).

The changes in the composition of cytoplasm and membranes provide a biochemical basis for plant acclimation to cold. The acclimation of plants to cold is usually connected with a significant growth inhibition and evocation of dormancy (in deciduous woody plants; Sakai and Larcher 1987, Renault *et al.* 2005, Kalberer *et al.* 2006). The process of cold acclimation includes two major aspects: the adjustment of metabolism and basic cellular functions to biophysical constraints imposed by low temperature (LT), and the induction of frost tolerance (FT) (often expressed as a lethal temperature when 50 % of the sample die - LT_{50}). The first aspect differentiates the chilling-sensitive species from the chilling-tolerant ones while the second aspect of cold acclimation discriminates the chilling-tolerant, but freezing-sensitive species from those which are freezing-tolerant (Guy 1990). Non-acclimated rye, for instance, is killed by freezing at about -5 °C, but after a period of exposure to low non-freezing temperature can survive freezing down to about -30 °C (Thomashow 1999). At least two factors other than LT exposure which can induce FT have been previously described: the application of exogenous ABA at concentrations between 10^{-4} and 10^{-5} M (high non-physiological concentrations), and controlled plant tissue desiccation. While it is known that in desiccated plant tissue the concentration of endogenous ABA increases it is very likely that both pathways are interconnected (Sakai and Larcher 1987, Guy 1990). The maintenance of high FT during the winter is controlled by several genetic mechanisms. Among them, vernalization genes in cereals and photoperiodically activated genes present some of currently most studied mechanisms connected with the maintenance of winter hardiness (Fowler *et al.* 2001, Welling *et al.* 2004). It also becomes obvious that the actual level of FT in plants has a close relationship to their developmental stage (Prášil *et al.* 2004).

The transition from cold-acclimated to non-acclimated state (deacclimation) is usually associated with substantial developmental changes, renewed plant growth and renewed cell hydration (Kalberer *et al.* 2006). Deacclimation is generally more rapid than cold acclimation. Whereas the gaining of a maximum FT by cold acclimation often affords several weeks of LT-treatment, the loss of FT can occur during a few days of sufficiently high temperatures. However, freezing-tolerant plants usually retain some level of FT and can rapidly reacclimate after the return of low temperatures (Guy 1990).

The winter hardiness, *i.e.*, the ability of plants to survive winter, has more components than just the FT. For instance, desiccation tolerance, tolerance to the

effects of snow cover or tolerance to flooding present other important components of winter hardiness because the winter is usually characterised not only by severe frost, but also by freeze-thaw cycles in some areas.

In the following sections, we focus on the role of dehydrins in a cold response in selected herbaceous dicotyledons, in some woody species, and the dominant interest will be paid to the role of dehydrins in a cold response of cereals.

The role of dehydrins in the cold response of herbaceous dicotyledons

The herbaceous perennials, biennials, but also the annuals have to cope with the seasonal effects of cold, especially in the early spring, during their individual development. Expression and accumulation of dehydrin proteins presents one important component of their protection against cold stress. Currently, identification and characterization of dehydrins induced by cold has been conducted in many species (Table 1).

In *Arabidopsis thaliana*, six dehydrin genes and four additional expressed sequence tags (ESTs) have recently been characterised (Puhakainen *et al.* 2004a). Of the *Arabidopsis* dehydrin genes, *Cor47* (Gilmour *et al.* 1992, Wellin *et al.* 1995, Iwasaki *et al.* 1997), *Rab18* (Lang and Palva 1992), *Lti29* (*ERD10*) (Wellin *et al.* 1994, 1995), *Lti30* (*DHNXero2*) (Wellin *et al.* 1994), and *ERD14* (Kiyosue *et al.* 1994) have been reported to being up-regulated under cold stress. Nylander *et al.* (2001) observed a different pattern of protein accumulation and tissue localisation under various stresses. COR47 and LTI30 were the major cold-induced dehydrins. The highest accumulation of cold-induced dehydrins was observed in vascular tissue. In addition to the vascular tissue, ERD14 and LTI29 were also present in root tips of unstressed plants. Puhakainen *et al.* (2004a) observed enhanced FT (expressed as LT_{50} values) in transgenic *Arabidopsis* plants over-expressing two double dehydrin gene constructs under the CaMV 35S promoter: pTP9 containing *Rab18* and *Cor47* and pTP10 containing *Lti29* and *Lti30*. Using the immunolocalisation technique, the authors showed that the improvement of FT in TP10 lines was due to the association of acidic dehydrin LTI29 with plasmalemma and membranaceous vesicles in the cytoplasm in *Arabidopsis* cells. The promoter region of *Lti30* was analysed by Rouse *et al.* (1996) using a promoter fusion with the β-glucuronidase (GUS) reporter gene. The expression of GUS under *Lti30* promoter was induced by ABA, wounding, cold and dehydration and the gene product was detected in desiccated seeds and pollen grains, in young seedlings, in roots (with the exception of the root tip), in trichomes and the vascular tissues of leaves and stems in mature plants.

Alsheikh *et al.* (2003) found that acidic dehydrin ERD14 undergoes phosphorylation of several serine residues in its serine tract upon cold, which is mediated by cold-regulated kinases. Phosphorylated ERD14 possesses a calcium-binding activity. It was found to be localised in the cytosol near the plasmalemma. It has been known for a long time that the cellular responses to many environmental stresses including cold is mediated by signalling pathways involving Ca^{2+}. Generally, cytosolic concentrations of Ca^{2+} are extremely low (around 200 nM) and increase by several orders during signalling as a consequence of regulated transport from the apoplast, vacuole and endoplasmic reticulum. Calcium then binds to several specific proteins, *e.g.*, calmodulin which then alters the activity of other proteins. Thus, ERD14 phosphorylation and its Ca^{2+}-binding activity seems to be specifically induced by cold stress. The authors proposed that ERD14 possessing bound Ca^{2+} may have a function of ionic buffer or sugar chaperone under cold stress similar to calreticulin or calnexin that bind Ca^{2+} in the endoplasmic reticulum. But they concluded that this hypothesis has not yet been tested. Recently, Alsheikh *et al.* (2005) showed that *in vitro* phosphorylated COR47 and ERD10 are also able to bind Ca^{2+} and therefore it can be proposed that a Ca^{2+}-binding activity is a trait shared by acidic dehydrins in *A. thaliana*.

In *Brassica napus* and *B. juncea*, dehydrin genes named *BnDHN1* and *BjDHN1* were identified by Yao *et al.* (2005) by the cloning of cDNA sequences. Both genes encode Y_3SK_2 dehydrins and share 100 % nucleotide identity according to their cDNA sequence (the probable cause is the fact that both *Brassicacae* species are allotetraploid and share the A genome). It was shown by the authors that these genes are expressed only in germinating seeds and that they enhance the seed cold tolerance during seedling emergence. Surprisingly, no *BnDHN1* or *BjDHN1* mRNAs were detected in dry seeds.

Recently, another ABA- and cold-induced dehydrin gene was detected in *B. napus* by Deng *et al.* (2005). Other dehydrin genes have also been identified in *Capsella bursa-pastoris* (Fan and Wang 2006).

In *Solanum tuberosum*, Kirch *et al.* (1997) have identified a stress-induced dehydrin gene *ci7*. Its expression is induced by cold (4 °C), drought, high salinity and exogenous ABA. It is notable that the protein was detected only in tubers upon stress treatments listed above while it was absent in leaves under the same conditions.

In the wild potato (*Solanum sogarandinum*) Rorat *et al.* (2006) detected significant levels of DHN24 in transporting tissues, in apical parts, and in tubers under normal growth conditions whereas no DHN24 was detected in leaves. Additionally, in *S. tuberosum* and *S. sogarandinum*, a KS-type dehydrin named DHN10 was detected in significant amounts in tubers, stems and flowers of non-stressed plants by Rorat *et al.* (2004). The abundance of DHN10 depends on organ type and age. During LT-treatment (4 °C), the DHN24 protein content

substantially increased in tubers, in transporting organs and in apical parts, and only a small increase was observed in leaves. Contrary to DHN24, the amount of DHN10 increases in mature leaves under cold conditions. It should be emphasised that the increase in protein abundance (both DHN24 and DHN10) was observed only in the plants that were able to cold acclimate and it correlated with their acclimation capacity. These results suggest that the expression of both *Dhn24* and *Dhn10* are regulated by organ-specific factors under control conditions and by both organ specific and stress factors in mutual collaboration under stress conditions. It was also shown by Yin *et al.* (2006) that transgenic cucumber plants (*Cucumis sativus*) cv. Borszagowski of the line TCH10 exhibited enhanced FT when expressing the DHN24 protein from *S. sogarandinum* under cold stress (4 °C).

In freezing-tolerant *S. commersonii* and freezing-sensitive *S. tuberosum* cv. Bintje, two homologous dehydrin genes *Scdhn1* and *Stdhn1*, have been identified by Baudo *et al.* (1996). It was demonstrated by the investigators that they are expressed in response to cold and ABA.

In *Spinacia oleracea*, a cold-induced dehydrin CAP85 was identified by Neven *et al.* (1993). It has 11 copies of the K-segment within its molecule and exhibits a significant cryoprotective activity using lactate dehydrogenase (LDH) assay.

In *Medicago sativa*, a dehydrin named CAS15 was characterised by Monroy *et al.* (1993) in response to cold. The authors observed that the accumulation of CAS15 is associated with enhanced hardening capacity in *M. sativa*. Similarly, in cell suspension cultures of *M. falcata*, a dehydrin CAS18 was identified by Wolfraim *et al.* (1993) upon cold treatment.

Dehydrins were also identified in some chilling-sensitive tropical and subtropical legume crops where they are induced by many stress factors including cold.

In *Cicer pinnatifidum*, a wild relative of important tropical and subtropical crop *C. arietinum*, a dehydrin gene named *cpdhn1* was identified by Bhattarai and Fettig (2005) from a cDNA library. The dehydrin protein, CpDHN1, accumulates in seeds during their maturation and it was also detected within leaves in response to drought, chilling (4 °C), salinity, ABA and methyl jasmonate treatment (for detail see Table 1). The induction of *cpdhn1* expression by ABA and methyl jasmonate suggests that this dehydrin may be induced by biotic stress factors. The expression of CpDHN1 protein may thus improve the tolerance of *C. arietinum* to a wide variety of environmental stress factors, both abiotic and biotic.

In *Vigna unguiculata*, an extremely chilling-sensitive annual crop, a 35-kD protein enables young seedlings to emerge successfully under cold conditions in the field (Ismail *et al.* 1999b). Otherwise, soil temperatures below 20 °C can cause significant inhibition of seedling emergence. The 35-kD protein present in the seeds of the cold-tolerant line 1393-2-11 was purified and described as DHN1. It was shown that its presence in mature seeds of cowpea co-segregated with chilling tolerance during seedling emergence (Ismail *et al.* 1999b).

The role of dehydrins in the cold response of woody plants

Recently, several studies have shown that the accumulation of dehydrins and other stress proteins also plays an important role in the acclimation of woody plants to unfavourable temperatures (Table 1).

In flower buds of cold tolerant *Vaccinium corymbosum*×*Vaccinium darrowi*, 65, 60 and 14 kD dehydrins were detected by Muthalif and Rowland (1994) using the antibody against K-segment. It was shown by Levi *et al.* (1999) that the 65 and 60 kD dehydrins are *O*-glycosylated *in vivo*, *i.e.*, they undergo a post-translational modification. The authors characterised a 2 kb-cDNA segment, identified as dehydrin of 60 kD and named the corresponding gene *bbdhn1*. A 14-kD dehydrin was further characterised by Dhanaraj *et al.* (2005) and named BbDHN6. In addition to 14-kD dehydrin, a new dehydrin of 16 kD (encoded by gene *bbdhn7*) was identified by the investigators. It was suggested that this protein may be induced by short photoperiods.

In cell cultures of blueberry cv. Gulfcoast, two dehydrins of 65 and 30 kD were detected on mRNA and protein levels by Parmentier-Line *et al.* (2002) in control plants. During a two-week treatment at 4 °C, the level of 65 kD dehydrin did not change significantly. However, the level of 30 kD dehydrin increased significantly after only 1 d at 4 °C and then increased gradually during the whole period of cold treatment.

In the leaves of young plants of *Populus tremula*×*Populus tremuloides* six prominent bands belonging to proteins of M_r 147, 80, 60, 36, 26, and 19 kD were detected using a specific antibody against poplar DHNs (Renault *et al.* 2005). Two bands belonging to DHNs of M_r 60 and 26 kD were present constitutively, although the amount of the 60-kD DHN increased significantly during the 2-week cold treatment. However, the other bands became detectable only in LT conditions (4 °C). Additionally, the intensity of bands increased under LT and the authors had proven that it strongly correlated with the increase in FT expressed as LT_{50} values of the plants.

In the *Prunus persica*, a dehydrin PCA60 of Y_2K_9 type with M_r of 60 kD encoded by the *Ppdhn1* gene was identified by Arora and Wisniewski (1994) and further purified and characterised by Wisniewski *et al.* (1999). It was found that PCA60 is localised within bark cells and xylem ray parenchyma cells in the cytoplasm, plastids, nucleus and nucleolus. A significant seasonal pattern of PCA60 expression was observed by Artlip *et al.* (1997) in

both a deciduous and an evergreen peach cultivars. PCA60 does not fall into any dehydrin subclass described by Close (1997). The protein shows a significant cryoprotective activity when using the LDH assay. In addition, PCA60 also exhibits a direct antifreeze activity, *i.e.*, it actively modifies the rate of growth of ice crystals and their final shape (Wisniewski *et al.* 1999). Recently, another LT- and SD-induced dehydrin gene, *Ppdhn3*, has been identified in peach by Bassett *et al.* (2006).

In citrus trees, dehydrins were first identified by Cai *et al.* (1995) in a cold-tolerant *Poncirus trifoliata*. The two cold-induced dehydrins of KS- type identified in *P. trifoliata* were described as COR11 and COR19. In *Citrus paradisi*, a dehydrin called COR15 was detected in peel tissue (flavedo) of mature fruits by Porat *et al.* (2002). Its expression enhances fruit chilling tolerance. It was found by the authors that the amount of *Cor* mRNA increases in chilled fruits after brief treatment with hot water (62 °C for 20 s) which preceded cold. This finding can help the breeders with the storage of citrus fruits. A dehydrin named CuCOR19 was detected in the leaves of *Citrus unshiu* (Hara *et al.* 1999). Its expression was induced by cold (4 °C) to significant levels whereas increased concentrations of ABA (0.1 - 10 µM) or NaCl (50 - 200 mM) affected it only very slightly. Hara *et al.* (2001) showed a significant cryoprotective activity of CuCOR19 using catalase (CAT) and LDH assays. CuCOR19 also exhibits a radical-scavenging function against liposome peroxidation. Overexpression of CuCOR19 under CaMV 35S promoter in transgenic tobacco enhanced its cold tolerance and prevented lipid peroxidation. The protein was predominantly localised in mitochondria of transgenic plants (Hara *et al.* 2003). The authors also showed that transgenic tobacco seeds accumulating CuCOR19 protein began to germinate earlier under cold when compared to wild-type plants. Later, Hara *et al.* (2004) reported that CuCOR19 can scavenge hydroxyl radical and peroxyl radical. The authors found out that this protein is rich in glycine, histidine and lysine residues which are potential targets of these radicals. The authors found that dehydrins in *Arabidopsis* have similar glycine, histidine and lysine contents to that of CuCOR19. Recently, Hara *et al.* (2005) have detected a new dehydrin in the flavedo tissue of *C. unshiu* which was named CuCOR15. The authors found a significant metal-binding activity for this protein which is provided by its histidine-rich domains. The accumulation of CuCOR15 is enhanced by cold stress. The metal-binding activity of CuCOR15 is probably associated with its antioxidative activity since free metal ions present an important catalytic agent for radical formation in the cells. Thus, the dehydrins may not only act as chaperones or cryoprotectants, but that they also can directly reduce lipid peroxidation and protein oxidation during cold and other abiotic stresses. This conclusion can significantly broaden our current knowledge of the role of dehydrins in the protection of proteins and membranes upon stress conditions.

All citrus dehydrins characterised above are very similar; they possess an unusual K-segment resembling the K-segment in gymnosperms and an S-segment at an unusual position at the C-terminus. They also have NLS. However, their function in the nucleus is not clear yet. The dehydrins of this type occur as multicopy genes in the citrus genome. Apart from these dehydrins, two dehydrin genes (*csDHN* and *cpDHN*) with the typical angiosperm-type K-segment were recently characterised by Porat *et al.* (2004) in *Citrus sinensis* and *C. paradisi*. The content of their mRNAs increase in chilled fruits after a brief hot water treatment. They are present only in one copy per genome.

In *Pistacia vera*, a dehydrin-like protein PV-DHN was detected by Yakubov *et al.* (2005). The corresponding gene, *PV-dhn*, is expressed during cold winter months, reaching the maximum in December and January when the maximum temperatures reach 20 °C and minimum only 0 °C in the Negev desert highlands. The protein accumulates predominantly in the outer leaves of the inflorescence buds and in the bark of stems of young trees. Immunogold labelling showed that it is a cytoplasmic protein with no specific organellar localization.

In various evergreen *Rhododendron* species, multiple dehydrins ranging from 25 kD to 73 kD were detected in the leaves. It was shown by Lim *et al.* (1999) that the amount of a 25-kD dehydrin accumulated in the leaves correlates with leaf FT in F_2 segregants of the cross between *R. catawbiense* and *R. fortunei* differing in their FT. It was concluded by Lim *et al.* (1999) and Marian *et al.* (2003) that the 25-kD dehydrin can be considered a marker of leaf FT in many *Rhododendron* species.

In *Betula pendula*, a dehydrin gene named *Bplti36* was isolated by Puhakainen *et al.* (2004b) from a cDNA library. The promoter of this gene contains five C-repeat, dehydration-responsive, and low temperature-responsive elements (CRT/DRE/LTREs) and one ABA regulatory element (ABRE). It was shown by the authors that the expression of *Bplti36* is up-regulated by cold, drought, salinity and exogenous ABA. It was also proven that the expression of *Bplti36* under synergistic LT and short day (SD) treatment was higher compared to LT or SD treatments alone, thus confirming the prerequisite that both LT and SD act as environmental signals inducing FT in silver birch under natural conditions. After the transfer of *Bplti36* promoter fused with the *uidA* reporter gene into transgenic *Arabidopsis* overexpressing C-repeat binding factor 3 (CBF3), the plants synthesized the reporter gene. Hence the authors verified the hypothesis that the CBF regulatory pathway is universal within higher plants. In *B. pubescens*, Rinne *et al.* (1999) found a dehydrin of 33 kD belonging to the Rab-16 family in the apices of non-cold-acclimated plants. Apart from this dehydrin, a 24-kD dehydrin was found to accumulate during cold acclimation in the nuclei, storage protein bodies and starch-rich amyloplasts during cold acclimation. The authors proposed that the association of this dehydrin with starch granules is due to its protective activity upon the enzymes of starch metabolism (dehydrin provides water necessary for enzyme function). They

actually proved a protective function of partially purified 24-kD dehydrin on the activity of α-amylase (EC 3.2.1.1.).

More recently, Welling *et al.* (2004) have characterised two dehydrins which are expressed during the winter dormancy in birch: BpuDHN1 which was found to be present in buds in autumn at the beginning of the dormant state, and BpuDHN2 which accumulates during the coldest winter months. The expression of *BpuDHN1* is regulated by both photoperiod and low temperature whereas the expression of *BpuDHN2* was predominantly affected by low temperature with a lesser contribution of the photoperiod.

The dehydrins and other stress-related proteins associated with the cold acclimation of woody plants were observed in the bark tissues of eight species *Prunus persica, Malus domestica, Rubus* sp., *Populus nigra, Salix babylonica, Cornus florida, Sassafras albidum,* and *Robinia pseudo-acacia* by Wisniewski *et al.* (1996). These authors detected a considerable increase in dehydrin accumulation during the winter and a subsequent decrease in the spring in all species used in the study although significant differences were observed between them. In *P. persica* cv. Loring the same 60-kD dehydrin was observed which was previously detected in an unrelated cultivar of peach (Arora and Wisniewski 1994). The greatest diversity in DHNs was observed in black locust. In poplar and willow, a similar dehydrin pattern was observed during the year. In willow, three DHNs of M_r larger than 106 kD were detected whereas in blackberry and sassafras, several DHNs with M_r ranging from 25 to 30 kD were found. The M_r of other major DHNs were 47 kD in apple, 57 kD in willow, 80 and 45 kD in poplar, several bands ranging from 30 to 40 kD in black locust, from 60 to 70 kD in thornless blackberry, and from 30 to 50 kD in flowering dogwood.

Dehydrins have also been found in gymnosperm woody species using the anti-dehydrin antibody by Close *et al.* (1993). The gymnosperm K-segment consensus sequence is (Q/E)K(P/A)G(M/L)LDKIK(A/Q)(K/M)(I/L)PG while the angiosperm K-segment consensus sequence is EKKGIMDKIKEKLPG (Jarvis *et al.* 1996, Close 1997). In two-year-old seedlings of *Pinus sylvestris*, a 60-kD dehydrin was found by Kontunen-Soppela *et al.* (2000). The authors showed a decrease in the amount of this protein during seedling deacclimation in the spring. Nitrogen-fertilized seedlings showed a more rapid decrease in dehydrin content during dehardening compared to control ones since nitrogen-fertilization enhanced the renewed growth activity during dehardening. In *Picea glauca*, Richard *et al.* (2000) characterised a dehydrin gene named *PgDhn1* isolated from a cDNA which was shown to encode a 27-kD protein whose expression is induced by cold and drought treatments, upon wounding or by both jasmonic acid and methyl jasmonate treatments.

The role of dehydrins in the cold response of cereals

Rye, wheat and barley are closely related genetically. They all possess a basal set of 7 chromosomes, although the chromosomes can occur in multiple sets (in hexaploid wheat, for instance, three sets – A, B, and D genome are present). Barley is only diploid and possesses one H genome. Similarly, rye possesses one set of R genome. The expression of dehydrin genes in response to cold was predominantly studied in the two freezing-tolerant members of the *Triticae, i.e.,* wheat and barley. In addition to cold-induced dehydrins in *Triticae*, a LT-induced dehydrin gene, *OsDhn1*, has been identified in rice (*Oryza sativa*) by Lee *et al.* (2005) (Table 1).

Wheat: Two major groups of dehydrin genes induced by cold have been detected in wheat: the *Wcs120*, and the *Wcor410* (Fowler *et al.* 2001). Apart from these families, the K-segment is present in other gene families in wheat, *e.g.,* in *Rab* genes (Close 1997, Borovskii *et al.* 2002) which are not predominantly cold-inducible.

According to Sarhan *et al.* (1997), the WCS120 protein family includes 7 members with apparent M_r ranging from 12 to 200 kD: WCS200 (Mr 200 kD), WCS180 (180 kD), WCS66 (50 kD), WCS120 (50 kD), WCS40 (40 kD), WCS726 (21 kD), and WCS80 (12 kD). *Wcs120* genes encoding high-M_r WCS120 proteins (WCS200, WCS66, and WCS120) are located on homoeologous group 6 chromosomes. It was shown by Ohno *et al.* (2003) that *Wcs726* (*Wcor726*) shares a 93 % nucleotide sequence homology with a small member of *Wcs120* family known as *Wdhn13*. It has been proposed that these two genes are identical. Similarly, a K_6 dehydrin of 39 kD was found in wheat by Guo *et al.* (1992) and was described as COR39. Its characteristics are very similar to WCS120 and it can be hypothesized that these two proteins are identical. With other dehydrins, the WCS120 protein family shares only multiple copies of the K-segment (the proteins belong to the K_n subclass of dehydrins) whereas no Y- or S-segments can be found in these molecules (Sarhan *et al.* 1997).

During cold acclimation, the WCS120 proteins accumulate predominantly in the meristematic tissues because the survival of these tissues is crucial for the survival of the whole plant in the winter. The WCS120 protein possesses a relatively high cryoprotective activity (PD_{50} of 10 μg cm^{-3}) in protecting the enzymatic activity of LDH. Therefore, it can be concluded that the WCS120 protein acts as an important protective agent of many vital cellular proteins in cold-acclimated plant tissue (Houde *et al.* 1995, Sarhan *et al.* 1997). Since WCS120 proteins are exclusively LT-inducible, *i.e.,* they are not present in wheat tissues under favourable growth temperatures, they can be considered a marker of FT (Houde *et al.* 1992).

The *Wcor410* gene family has three homologous members *Wcor410a, Wcor410b,* and *Wcor410c* which are located on the long arm of the homoeologous group

6 chromosomes of hexaploid wheat. The WCOR410 proteins are highly hydrophilic, acidic dehydrins of the SK_3 type which have been found to be localised near the plasmalemma (Danyluk *et al.* 1994, 1998). A positive correlation between the accumulation of *Wcor410* transcripts and the capacity of different wheat cultivars to develop FT was found by Danyluk *et al.* (1994). Later, Houde *et al.* (2004) transferred the *Wcor410a* gene into the strawberry and reached a 5 °C improvement of FT of the transgenic leaves over both the wild-type (WT) leaves and transformed leaves not expressing the WCOR410 protein under cold. However, no effect of transformation on FT was observed upon normal growth temperature suggesting that the synthesis of WCOR410 is activated only upon LT.

Recently, a KS-type LT-induced dehydrin gene *Wcor825* was found in the wheat genome (Accession Number T06808).

Barley: In barley, 13 dehydrin genes have been identified recently (Choi *et al.* 2000, Rodriguez *et al.* 2005). They are located on chromosomes 3H, 4H, 5H, and 6H and they differ in their M_r, pI, and induction conditions of their expression (for cold and mild frost-induced dehydrins, see Table 1).

In response to cold (2 - 4 °C), the expression of DHN5 was detected on immunoblots using the anti-dehydrin antibody developed by Close *et al.* (1993). Its M_r on immunoblots ranges from 80 to 86 kD (Van Zee *et al.* 1995, Bravo *et al.* 1999) though its M_r calculated from its amino acid sequence is only 58.5 kD (Close *et al.* 1995). This discrepancy between actual M_r of DHN5 and M_r on SDS gels is also typical for other dehydrin proteins and is caused by their unique amino acid composition (see Introduction). It has been proven by Van Zee *et al.* (1995) that an 86-kD dehydrin is DHN5 using purified recombinant barley DHN5 isolated from *E. coli* because both proteins co-migrated on the gels. Bravo *et al.* (2003) used the amino acid analysis of P-80 and the analysis of proteolytic fragments of P-80 and DHN5 by reverse phase chromatography to ensure that P-80 and DHN5 share more similarities than expected for two different proteins. DHN5 (K_9) shows a sequence homology to wheat WCS120 (K_6) which is the major cold-induced dehydrin in wheat. Both are also located on homoeologous group 6 chromosomes. It has been reported by some authors (Zhu *et al.* 2000) that DHN5 accumulates in larger amounts in freezing-tolerant cv. Dicktoo than in freezing-sensitive cv. Morex, whereas other authors (Van Zee *et al.* 1995) observed no significant differences in the accumulation of DHN5 between tolerant and sensitive barley cultivars. Bravo *et al.* (1999) found also a correlation between the accumulation of DHN5 and LT_{50} values in three barley cultivars and concluded that the accumulation of DHN5 is associated with the induction of FT in all three cultivars during cold acclimation. It should be noted that the amount of DHN5 recognized by the antibody in 6 d-cold-treated plants of cv. Aramir completely disappeared after 6 d following the transfer of plants to higher non-inducing temperatures, *i.e.*, a deaccli-mation treatment (Bravo *et al.* 1999). Bravo *et al.* (2003) also detected a significant cryoprotective activity of DHN5 using a LDH assay.

Apart from a strong band belonging to DHN5, Bravo *et al.* (1999) observed several minor bands with M_r lower than DHN5 in three barley cultivars after 30 d of cold treatment. The bands were strongly developed especially in the cv. Frontera. These weaker bands which have remained unidentified have been also observed by us in both the spring cv. Atlas 68 and winter cv. Igri after at least two weeks of cold (Kosová *et al.*, unpublished). It can be suggested that these polypeptides are somehow derived from DHN5, but the hypothesis that DHN5 can undergo an alternative splicing should be rejected because no intron has been described in the primary amino acid sequence of DHN5 (Close *et al.* 1995). It was proposed by Nylander *et al.* (2001) who observed a minor band of ERD14 on their blots using specific anti-ERD14 antibody that these two proteins could differ in their N-terminal regions, *i.e.*, that the minor band presents a product of alternative AUG initial codon usage during initiation of translation. These bands of lower M_r could arise by similar mechanisms.

Apart from *Dhn5*, the expression of *Dhn8* (an acidic SK_3 dehydrin, homolog of wheat WCOR410; both are located on homologous 6 chromosomes) has been reported by Zhu *et al.* (2000) on the transcript level using reverse transcriptase polymerase chain reaction (RT-PCR) under cold conditions. However, its expression was later induced by cold (4 °C), but was weaker when compared to *Dhn5*.

Mild frost (-2 °C or -4 °C) followed by cold (4 °C) resulted in the induction of other *Dhn* genes and low-M_r proteins according to Zhu *et al.* (2000). Using specific RT-PCR primers, transcripts of *Dhn1, Dhn2, Dhn3, Dhn4, Dhn7* and *Dhn9* were detected in this experiment. *Dhn1, Dhn2* and *Dhn9* are located on chromosome 5H near QTL for winter hardiness.

Recently, a small KS-type dehydrin, *Dhn13*, was found by Rodriguez *et al.* (2005) on chromosome 4H. It was shown by the researchers that its expression is constitutive although it increases significantly upon abiotic stress conditions (2.8-fold upon cold and 8.5-fold upon mild sub-zero temperatures). It was also found by the authors that its sequence is similar to wheat *Wcor825*.

Important regulatory mechanisms involved in dehydrin expression during cold

A plant's direct response to cold can be mediated by several ABA-dependent and ABA-independent mechanisms (Thomashow 1999, Yang *et al.* 2005). It was shown by Lang *et al.* (1994) that upon LT, the content of endogenous ABA increases transiently. Many dehydrin and other cold-regulated structural genes contain ABRE

elements in their promoters. The ABREs possess two fragments: TACGTCC (the G-box) and GGCCGCG (GC-motif). It is known that bZIP transcription factors interact with ABRE elements (Thomashow 1999, Allagulova *et al.* 2003). It was shown that the expression of *Arabidopsis* dehydrin gene *Rab18* which contains ABRE elements in its promoter is enhanced in response to cold. Lang *et al.* (1994) found out that ABA-deficient (*aba-1*) and ABA-insensitive (*abi1*) mutants of *Arabidopsis* are not able to develop sufficient level of FT comparable with WT upon LT treatment. However, the content of endogenous ABA increases markedly less (only 2- to 3-fold compared to control) upon LT than upon drought treatment.

A well-investigated ABA-independent LT-induced regulatory pathway is mediated by *CBF* transcriptional activators. They bind to a CRT/DRE/LTRE *via* its AP2 domain (a DNA binding motif). Three cold-inducible CBF transcriptional factors – *CBF1*, *CBF2*, and *CBF3* binding to CRT/DRE/LTRE have been characterised in the genome of *Arabidopsis* (Gilmour *et al.* 2004) while *CBF4* is inducible by ABA and drought but not by cold (Yang *et al.* 2005). The CRT/DRE/LTRE sequence is present in the promoters of many cold-induced structural genes and contains the 5-bp core sequence CCGAC (Thomashow 1999). For instance, the promoter of *Wcs120* gene contains two CRT/DRE/LTREs to which a wheat ortholog of *Arabidopsis* CBF1 is supposed to bind (Sarhan and Danyluk 1998). It has also been proposed that the ortholog of CBF1 requires certain activators or adaptors for its binding to a CRT/DRE/LTRE element. It has been suggested by Vazquez-Tello *et al.* (1998) that specific kinases and phosphatases may modify the activity of this transcription factor during cold acclimation. An ortholog of *Arabidopsis CBF3* gene was identified in barley on chromosome 5H near QTL for winter hardiness by Choi *et al.* (2002) and named *HvCbf3*.

However, a successful induction of a high level of FT in *Arabidopsis* plants caused by the over expression of the *CBF1* gene shows a possible way how to solve a tough task: to improve FT of important agronomical crops. FT is a multigenic trait, but simultaneous successful transformation of a higher number of genes may be very difficult. Thus, a direct manipulation with a transcription factor may lead to a desirable effect. Jaglo-Ottosen *et al.* (1998) managed to enhance the FT in *Arabidopsis* plants by the overexpression of *CBF1*. It should be noted that as a consequence of enhanced *CBF1* activity, an increased expression of four *Cor* genes was detected in *Arabidopsis* plants. Similarly, the overexpression of *CBF3* led to the increase in FT characterised by enhanced expression of several *Cor* genes in transgenic *Arabidopsis* (Gilmour *et al.* 2000).

The induction, maintenance, and cessation of dehydrin expression and accumulation during the cold acclimation correlates with plant FT (Bravo *et al.* 1999, Fu *et al.* 2000, Fowler *et al.* 2001, Stupnikova *et al.* 2001, Renault *et al.* 2005) and could be regulated during development. It should be noted that only vegetative organs can significantly increase the actual FT under inducing environmental conditions while the generative usually can not (Sakai and Larcher 1987). Some plants have to undergo a certain period of LT before they switch their individual developmental programme from vegetative to reproductive phase. The requirement of LT is genetically inherited and is called vernalization. Genes responsible for the regulation of vernalization are named vernalization (*Vrn*) genes. The role of *Vrn* genes is intensively studied in cereals because they could be responsible for the differences in FT between spring and winter cultivars (Sarhan *et al.* 1997, Fowler *et al.* 2001, Prášil *et al.* 2005). Using a set of wheat reciprocal substitution lines in chromosome 5A, where the major *Vrn* gene is located, between the freezing-tolerant winter cv. Cheyenne and the freezing-sensitive spring cv. Chinese Spring, Limin *et al.* (1997) showed that the substitution led to the substantial increase in FT and WCS120 protein accumulation in the substitution line derived from Chinese Spring.

Relationships between *Vrn* genes, *Fr* genes (frost resistance genes which are located on homoeologous group 5 chromosomes) and the expression of CBF-regulated genes such as dehydrin genes are intensively studied in cereals (Danyluk *et al.* 2003, Kobayashi *et al.* 2005). The different regulation of *Vrn* gene expression could be one of the major causes of the different dynamics of FT development in spring and winter cultivars, involving the level of dehydrin expression and accumulation. A positive effect of dehydrin accumulation on the overwintering of young wheat plants was found by Stupnikova *et al.* (2002) in a frost-resistant winter wheat cv. Irkutskaia ozimaia under field conditions of eastern Siberia. A good correlation between dehydrin accumulation and FT was observed by Fowler *et al.* (2001) in the winter barley cv. Dicktoo during 10 weeks of cold acclimation (a time necessary for the fulfilment of vernalization requirement determined as the final leaf number). Significant differences in dehydrin accumulation between winter wheat cv. Norstar and spring wheat cv. Katepwa were observed by Fu *et al.* (2000) during 7 weeks of cold treatment. After 7 weeks of cold, dehydrins were nearly absent in Katepwa whereas in Norstar they accumulated to significant amounts. Similarly, differences in the accumulation of WCS120 proteins and FT between spring and winter wheat cultivars were observed by Stupnikova *et al.* (2001) after 9 d of LT-treatment (4 °C). However, significant differences in FT during a long-term cold acclimation were observed not only in winter *versus* spring cultivars, but also among various winter cultivars. The winter cultivars differing in their FT vary also in dehydrin content. Vítámvás *et al.* (2006) distinguished three-week-cold-acclimated winter wheat cvs. Mironovskaya 808 and Bezostaya 1 differing in their ability to develop FT on the basis of different accumulation of WCS120. Moreover, the winter cultivars with higher FT induced WCS120 proteins under higher temperature conditions (17 °C) more than lower-FT winter or spring cultivars (9 or 4 °C)

(Vítámvás *et al.*, unpublished). It seems that wheat cultivars with different levels of FT have different threshold temperatures for the induction and accumulation of WCS120 proteins.

Currently, our laboratory team has been investigating the dynamics in dehydrin accumulation in the winter barley cv. Igri and spring barley cv. Atlas 68 during 16 weeks of cold acclimation. The cv. Atlas 68 showed a rapid increase in dehydrin accumulation during the beginning of cold treatment followed by a slow decrease in the rest of the treatment. Contrary to cv. Atlas 68, cv. Igri showed a slow increase in dehydrin accumulation at the beginning of cold treatment and the maximum of FT and dehydrin accumulation is reached later. We have found out that when the cold-acclimated plants reach their maximum FT, the amount of dehydrins decrease although cv. Igri can retain significant amount of dehydrins after 16 weeks of cold treatment (Kosová *et al.*, unpublished).

Apart from the genetically inherited vernalization requirement, some plant species of high latitudes have evolved a different mechanism for induction of sufficiently high FT during winter - a photoperiodically activated development of FT. Since the winter is always signalled by SDs in high latitudes, a certain photoperiod can act as a signal inducing the development of FT and transition to the dormancy state. This mechanism is well characterised in deciduous trees, *e.g.*, silver birch. SDs induce the expression of genes responsible for the dormancy state. It was mentioned above that SDs also enhance the expression of some LT-induced genes, *i.e.*, dehydrins (Puhakainen *et al.* 2004b). The photoperiodic signal is probably sensed by phytochrome A (Welling *et al.* 2002).

Photoperiodic signal also plays an important role in the FT of winter cereals. The SD signal helps to maintain the LT-induced genes (dehydrins belong to them) involved in the development of FT in an up-regulated state for a longer time compared to LDs under low temperature treatment. The expression of the SD-induced genes involved in the development of FT is regulated by photoperiod (*Ppd*) genes (Fowler *et al.* 2001).

Methods of functional genomics used in cold stress research

Plant tolerance to cold and frost as an important component of winter hardiness attracts the interest of many plant physiologists, molecular biologists and also plant breeders due to its impacts upon the survival of many agronomical crops and other economically important plant species during winter and early spring. Plant response to cold presents a highly complex process in which many genes are involved. New methods of functional genomics, (*e.g.*, microarray analysis or two dimensional difference gel electrophoresis - 2D-DIGE) can provide useful tools for solving such problems.

Microarrays contain oligonucleotide sequences from a wide range of genes known in a given organism (*e.g.*, *Arabidopsis*) and are based on hybridisation between these oligonucleotides and cDNAs originating from mRNAs isolated from a sample (*e.g*,. cold-treated plant). Using this method, cold-induced or cold-repressed genes, for example, can be detected in a given plant species under specific conditions (by comparison with a control plant sample). Seki *et al.* (2002) designed a cDNA microarray covering about 7000 independent cDNA clones of *Arabidopsis* and observed the impact of cold, drought and salt stresses on gene up- or down-regulation. The researchers found 53 cold-, 277 drought- and 194 NaCl-inducible genes. However, they also detected a significant overlapping between these stresses, *i.e.*, a significant number of genes were up-regulated by two or even all three different stress factors. Among the genes up-regulated by these stress factors, 9 *Lea* transcripts including the dehydrins *ERD10*, *Cor47* and *Rab18* were detected. Similarly, up-regulated dehydrin genes have recently been found in cold-treated *Arabidopsis* by Fowler and Thomashow (2002), Maruyama *et al.* (2004), Hannah *et al.* (2005) and others. In winter wheat, up-regulation of the *Wcs120* gene family was detected by Gulick *et al.* (2005). A barley microarray was designed by Close (2005) which enables to do similar experiments on this important crop.

Another complex transcriptomic approach is represented by comparative studies of ESTs isolated from cold-acclimated and non-acclimated plant tissues. Using this method, major genes responsible for plant cold hardiness can be detected and further characterised. Wei *et al.* (2005) compared cDNA libraries from cold-acclimated versus non-acclimated leaf tissues of *Rhododendron catawbiense* and found four gene families that were highly abundant in cold-acclimated samples. They include dehydrins, early light-inducible proteins (ELIPs), and cytochrome P450 genes. Other examples of EST sequencing experiments on cold-treated woody plants are reviewed by Welling and Palva (2006).

2D-DIGE is a novel method in proteomics. It has several advances compared to normal two dimensional electrophoresis (2DE) technology: three different samples each bound to a different fluorescent dye can be separated and detected on one gel. It improves reproducibility of separation by reducing the variability between individual gels. It enables the researchers to detect a very wide range of proteins on one gel, especially to do a quantitative analysis of protein spots, as the intensity of the spot (protein conjugated with a dye) detected by a special scanner is proportional to the amount of the protein in the gel (Renault *et al.* 2006). Currently, Amme *et al.* (2006) have done an analysis of LT-induced (6 °C) proteins in *Arabidopsis*. They found 18 spots with at least 2-fold increased intensity compared to samples from plants grown at 10 °C; three of the spots were identified as dehydrins.

Current conclusions and future perspectives

In the identification of plant species tolerant to cold and frost, two major ways can be employed. First, there are the methods of classical breeding based on the selection of cultivars (genotypes, lines) possessing a given marker of FT (*e.g.*, expressing some cold-induced protein under selected temperature conditions, having a given level of FT after a given period of cold acclimation under certain temperature). Second, it can be proposed that the methods of genetic engineering will be employed in the improvement of FT of some crops of high economical interest. These methods will be based on detailed knowledge of the induction of FT in these crops and will be based on direct manipulations and alterations in gene expression of the genes participating in the induction and maintenance of FT in these crops. Several attempts have already been conducted in this area of research (*e.g.*, Jaglo-Ottosen *et al.* 1998, Hara *et al.* 2003, Houde *et al.* 2004, Yin *et al.* 2006). Dehydrins will certainly belong to the genes of interest due to their unique properties. In addition to their functions as emulsifiers, chaperones and cryoprotectants known for quite a long time, new functions have been reported recently for some members of dehydrin family – an antifreeze activity for PCA60 in peach, a calcium-binding activity for ERD14 in *Arabidopsis* and a metal-binding and radical-scavenging activity for CuCOR19 and CuCOR15 in *Citrus unshiu*. It is therefore certain that dehydrins remain an integral part of cold research.

Table 1. List of dehydrins induced by cold in selected plant species. The genes and proteins are characterised by corresponding accession numbers in NCBI (August 2006). M_r and pI were calculated from complete protein sequences using *ExPASy* (*Swiss Prot*). M_r determined empirically (by SDS-PAGE) are in brackets. AA - amino acid; Cys - cysteine, Gln - glutamine, Glu - glutamic acid, Gly - glycine, His - histidine, Lys - lysine. Cited references refer to the genes and proteins described, but not necessarily to other characteristics in all cases.

Organism	Gene (gene accession number)	Protein (protein type) (protein accession number)	Number of AA	M_r [kD]	pI	Other characteristics	Type of cold treatment	Reference
Arabidopsis thaliana (L.) Heynh.; thale cress	*Cor47* (AB004872)	COR47 (SK_3) (BAA23547)	265	29.9	4.75	2 CRT/DRE/LTRE and 1 ABRE in promoter	4/2 °C (day/night)	Wellin *et al.* 1995, Iwasaki *et al.* 1997
	Rab18 (X68042)	RAB18 (Y_2SK_2) (CAA48178)	186	18.5	7.10	accumulates in stomatal guard cells, predominantly in mature seeds	4/2 °C (day/night) + ABA	Lang and Palva 1992
	Lti29/ERD10 (X90958)	LTI29/ERD10 (SK_3) (CAA62448)	260	29.4	5.12	in root tips	4/2 °C (day/night)	Wellin *et al.* 1994, 1995
	Lti30/Xero2 (X77613)	LTI30 (K_6) (CAA54704)	187	20.1	8.95	in vascular tissues and anthers	4/2 °C (day/night)	Wellin *et al.* 1994
	ERD14 (D17715)	ERD14 (SK_2) (BAA04569)	185	20.8	5.41	in vascular tissues and bordering parenchyma; calcium-binding activity – possibly sugar chaperone	cold (4/2 °C day/night); expressed also in non-LT-treated plants	Kiyosue *et al.* 1994
Betula pendula Roth.; silver birch	*Bplti36*	BpLti36 (SK_2)		36	acidic	rich in Glu, 1Cys; 5 CRT/DRE/LTE and 1 ABRE in promoter	4 °C + SD	Puhakainen *et al.* 2004b
Betula pubescens Ehrh.; pubescent (downy) birch	*BpuDHN1* (AJ555331)	BpuDHN1 (Y_nK_n) (CAD87733)			basic	partial sequence	4 °C + SD	Welling *et al.* 2004
	BpuDHN2 (AJ555332)	BpuDHN2 (SK_n) (CAD87734)			acidic	partial sequence	4 °C, frost (-5 to -30 °C)	Welling *et al.* 2004
Brassica juncea (L.) Czern.; Indian mustard	*BjDHN1* (AY130999)	BjDHN1 (Y_3SK_2) (AAN08719)	183	19.2	6.67	expressed only in germinating seeds, not in dry mature seeds	5 °C	Yao *et al.* 2005
Brassica napus L.; oilseed rape	*BnDHN1* (AY303803)	BnDHN1 (Y_3SK_2) (AAQ74768)	183	19.2	6.67	expressed only in germinating seeds, not in dry mature seeds	5 °C	Yao *et al.* 2005

	BnERD10 (AY376669)	ERD10 (S_8K_2) (AAR23753)	271	31	5.09		cold + ABA	Deng *et al.* 2005
Capsella bursa-pastoris (L.) Medik.; shepard's purse	*Cbcor29* (DQ090957)	CbCOR29 (SK_3) (AAY84736)	261	29.4	4.93	typical SK_3 structure; homolog of COR47 in *A. thaliana*	cold	Fan and Wang 2006
Cicer pinnatifidum Jaub. and Spach	*Cpdhn1* (AY170010)	DHN1 (Y_2K) (AAN77521)	195	20.4	5.82		4 °C	Bhattarai and Fettig 2005
Citrus paradisi M.; grapefruit	*Cor15* (AY032975)	COR15 (K_2S) (AAK52077)	137	15.1	6.54	gymnosperm-type K-segment	cold (2 °C) after brief hot treatment (62 °C for 20 s) – in fruit flavedo	Porat *et al.* 2002
	cpDHN (AY160772)	cpDHN (SK_2) (AAN78125)	234	26.7	5.62	angiosperm-type K-segment	cold (2 °C) after brief hot treatment (62 °C for 20 s) – in fruit flavedo	Porat *et al.* 2004
Citrus sinensis [L.] Osbeck.; orange	*csDHN* (AY297793)	csDHN (SK) (AAP56259)	235	27.2	7.24	angiosperm-type K-segment	cold (2 °C) after brief hot treatment (62 °C for 20 s) – in fruit flavedo	Porat *et al.* 2004
Citrus unshiu Marcov.; Satsuma mandarin	*CuCOR15* (AB178479)	CuCOR15 (K_2S) (BAD97812)	137	15.2	6.54	metal binding and antioxidative activity	cold	Hara *et al.* 2005
	CuCor19 (AB016809)	CuCOR19 (K_3S) (BAA74736)	171	19	6.53	cryoprotective activity (LDH assay); radical scavenging activity	4 °C	Hara *et al.* 1999
Hordeum vulgare L.; barley	*Dhn1* (AF181451)	DHN1 (YSK_2) (AAF01689)	139	14.2	8.81	located on chromosome 5H near QTL for winter hardiness	sub-zero (-2 to -4 °C)	Choi *et al.* 1999, 2000
	Dhn2 (AF181452)	DHN2 (YSK_2) (AAF01690)	141	14.4	8.81	located on chromosome 5H near QTL for winter hardiness	sub-zero (-2 to -4 °C)	Choi *et al.* 1999, 2000
	Dhn3 (AF181453)	DHN3 (YSK_2) (AAF01691)	155	15.7	8.07	located on chromosome 6H	sub-zero (-2 to -4 °C) or cold combined with drought.	Choi *et al.* 1999, 2000
	Dhn4 (AF181454)	DHN4 (YSK_2) (AAF01692)	205	20.7	8.04	located on chromosome 6H	sub-zero (-2 to -4 °C) or cold combined with drought	Choi *et al.* 1999, 2000
	Dhn5 (AF181455)	DHN5 (K_9) (AAF01693)	575	58.5 (80)	6.65	located on chromosome 6H; homolog of wheat WCS120	5 °C	Close *et al.* 1995
	Dhn7 (AF181457)	DHN7 (YSK_2) (AAF01695)	191	19	9.10	located on chromosome 6H	sub-zero (-2 to -4 °C) or cold combined with drought	Choi *et al.* 1999, 2000
	Dhn8 (AF181458)	DHN8 (SK_3) (AAF01696)	255	27.7	5.21	located on chromosome 6H; „acidic dehydrin" - homolog of wheat WCOR410	5 °C	Choi *et al.* 1999, 2000
	Dhn9 (AF181459)	DHN9 (YSK_2) (AAF01697)	146	15.1	9.52	located on chromosome 5H	Sub-zero (-2 to -4 °C) or cold combined with drought	Choi *et al.* 1999, 2000
	Dhn13 (AY681974)	DHN13 (KS) (AAT81473)	107	12	6.84	located on chromosome 4H; in green tissues and anthers	Expressed also in non-LT-treated plants; enhanced by sub-zero (2/-10 °C day/night)	Rodriguez *et al.* 2005
Medicago falcata L.; alfalfa	*Cas18* (L07516)	CAS18 (AAA21185)	167	17,6	6,6	isolated from cold-acclimated cell suspension culture	5/2 °C (day/night).	Wolfraim *et al.* 1993
Medicago sativa L.; alfalfa	*Cas15b* (L12462)	CAS15 (K_2S) (AAA16926)	136	14.5	6.21		2 °C; enhanced by subzero (-2 °C)	Monroy *et al.* 1993
Oryza sativa L.; rice	*OsDhn1* (AY786415)	DHN1 (SK_3) (AAV49032)	290	30.9	5.68	acidic dehydrin; homolog of Wcs120 or Cor47	cold	Lee *et al.* 2005
Picea glauca (Moench.)Voss.; white spruce	*PgDhn1* (AF109916)	PgDHN1 (S_8K_4) (AAD28175)	245	27	6.9	amino acid composition analogous to wheat WCOR410	4 °C	Richard *et al.* 2000

Pistacia vera L.; pistachio	*PV-dhn* (Y07600)	PV-DHN (CAC34554)	230	25.9	7.1	rich in Gly and polar amino acids	0 - 20 °C	Yakubov *et al.* 2005
Poncirus trifoliata (L.) Raf.; trifoliate orange	*pBCORc119*	COR11 (KS)	106	11.4			4 °C	Cai *et al.* 1995
	pBCORc115 (S59536)	COR19 (K_3S)	179	19.8	6.9	rich in Gly, Gln, Lys, His, Glu	4 °C	Cai *et al.* 1995
Prunus persica (L.) Batsch.; peach	*Ppdhn1* (U62486 – clone *G10a*)	PCA60 (Y_2K_9) (AAC49658)	468	49.5 (60)	6.39	cryoprotective and antifreeze activity	cold	Artlip *et al.* 1997
	Ppdhn3 (DQ111949)	PpDHN3 (SK_2) (AAZ83586)	249	28,3	5,37	4 Cys residues in molecule	5 °C + SD	Bassett *et al.* 2006
Solanum commersonii Dun. ex Poir.; wild potato	*Scdhn1* (X83596)	ScDHN1 (CAA58575)	134	14.2	9.13		4/2 °C (day/night) + ABA.	Baudo *et al.* 1996
Solanum sogarandinum Ochoa; wild potato	*Dhn10* (AF542504)	DHN10 (KS) (AAN37899)	86	10	7.2	organ specific; developmental regulation	4 °C; expressed also in non-LT-treated plants.	Rorat *et al.* 2004
	Dhn24 (AY292655)	DHN24 (SK_3) (AAP44575)	210	23.8	5.25	organ specific; developmental regulation	4/3 °C (day/night); expressed also in non-LT-treated plants.	Rorat *et al.* 2006
Solanum tuberosum L.; potato	*Stdhn1* (X83597)	StDHN1 (CAA58576)	134	14.2	8.14		4/2 °C (day/night) + ABA.	Baudo *et al.* 1996
	ci7 (U69633)	Ci7 (SK_3) (AAB53203)	209	23.7	5.36	organ specific (tubers)	4 °C	Kirch *et al.* 1997
Spinacia oleracea L.; spinach	*Cap85* (M96259)	CAP85 (YK_{11}) (AAB88628)	535	61.5 (85)	5.94	cryoprotective activity	cold	Neven *et al.* 1993
Triticum aestivum L.; wheat	*Wcs200*	WCS200 (K_n) (AAB31285)		(200)	6.50	located on group 6 homoeologous chromosomes	6/2 °C (day/night).	Quellet *et al.* 1993, Limin *et al.* 1997
	Wcs180	WCS180 (K_n)		(180)	6.50	located on group 6 homoeologous chromosomes	cold	Houde *et al.* 1995, Limin *et al.* 1997
	Wcs66 (L27516)	WCS66/CS66 (K_7) (AAA21819)	469	46.8 (66)	6.74	located on group 6 homoeologous chromosomes	cold	Chauvin *et al.* 1994
	Wcs120 (M93342)	WCS120/CS120 (K_6) (AAA34261)	390	39 (50)	7.02	located on 6AL chromosome	4 °C	Houde *et al.* 1992
	Cor39 (AF058794)	COR39 (K_6) (AAC14297)	391	39 (50)	6.92	located on group 6 homoeologous chromosomes	2 - 18 °C	Guo *et al.* 1992
	Wcs40	WCS40 (K_n)		(40)	7.30		6/2 °C (day/night).	Houde *et al.* 1995
	Wcs726/ Wcor726 (U73213)	WCS726/ WCOR726 (K_n) (AAB18204)	124	12.7	7.04		cold	Danyluk and Sarhan 1996 (NCBI)
	Wcs80/ Wcor80 (U73212)	WCS80/ WCOR80 (K_n) (AAB18203)	93	9.6	8.05		cold	Danyluk and Sarhan 1996 (NCBI)
	Wdhn13 (AB076807)	WDHN13 (K_3) (BAC01112)	124	12.8	8.01	located on group 7 homoeologous chromosomes	4 °C	Ohno *et al.* 2003
	Wcor410a (L29152)	WCOR410 (SK_3) (AAA20189)	262	28	5.19	„acidic dehydrin"; located on group 6 homoeologous chromosomes	4 °C	Danyluk *et al.* 1994, 1998
	Wcor 410b (U73210)	WCOR410b (SK_3) (AAB18201)	268	28.8	5.25	homologue of WCOR410	4 °C	Danyluk and Sarhan 1996 (NCBI).
	Wcor410c (U73211)	WCOR410c (SK_3) (AAB18202)	259	27.9	5.2	homologue of WCOR410	4 °C	Danyluk and Sarhan 1996 (NCBI)
	Wcor825 (U73215)	WCOR825 (KS) (AAB18206)	73	8.1	8.08		cold	Danyluk and Sarhan 1996 (NCBI)
Vaccinium corymbosum L.; blueberry	*bbdhn1* (AF030180)	DHN1 (K_5) (AAB84258)	314	34.3 (60)	6.63	*O*-glycosylated; present in cold-hardy floral buds	0 - 7.2 °C	Levi *et al.* 1999

	Cor11/bbdhn7 (AY660960)	COR11 (K_2) (AAT76303)	108	11.8 (16)	7.97	present in stressed stems and leaves	cold	Dhanaraj *et al.* 2005
	Bbdhn6 (AY660959)	BbDhn6 (K_2) (AAT76302)	101	10.9 (14)	8.44	present in stressed stems and leaves	cold	Dhanaraj *et al.* 2005
Vigna unguiculata (L.) Walp.; cowpea	*Dhn1* (AF159804)	DHN1 (Y_2K) (AAF07274)	259	26.5 (35)	5.97	present in mature seeds	14 °C - in chilling-tolerant line 1393-2-11	Ismail *et al.* 1999b

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PAPER 3

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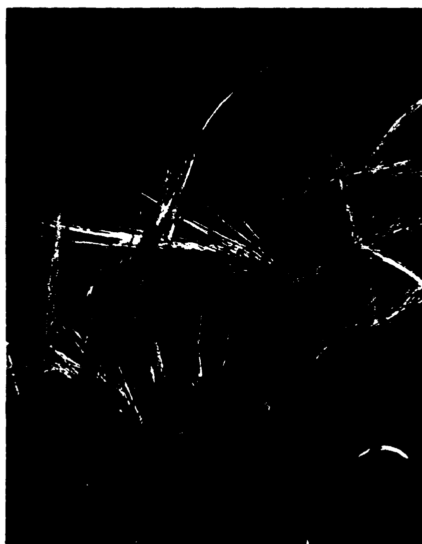

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Expression of dehydrin 5 during the development of frost tolerance in barley (*Hordeum vulgare*)

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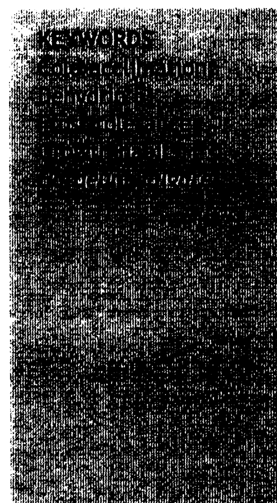

Summary

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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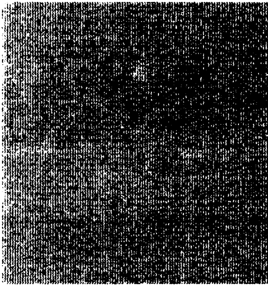

In contrast, no differences between the cultivars belonging to different growth habits in *Dhn5* mRNA accumulation were found. After 3 weeks of CA, the differences in accumulated DHN5 and FT between the individual growth habits became evident due to different developmental regulation of FT. The amount 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.

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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

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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.

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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[R] 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′-CCAAGGATCCACTT-GATGCT-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 (Semecký).

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

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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

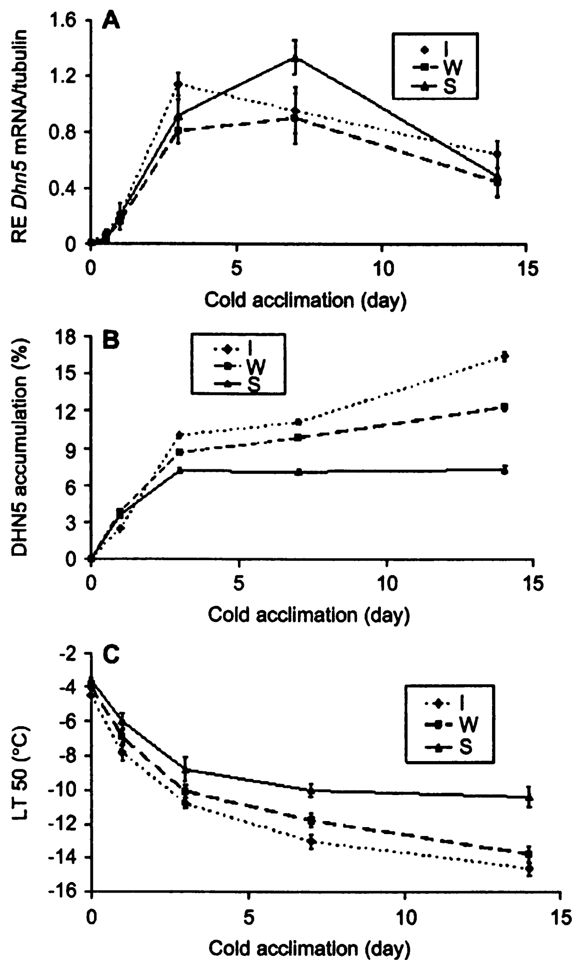

Figure 1. The kinetics of relative expression (RE) of *Dhn5* mRNA accumulation (A), DHN5 protein accumulation (B) and the dynamics of FT development (C) during the first 14 d of CA in 10 cultivars representing the three growth habits. The values of *I* are means from the values obtained on cultivars 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%.

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

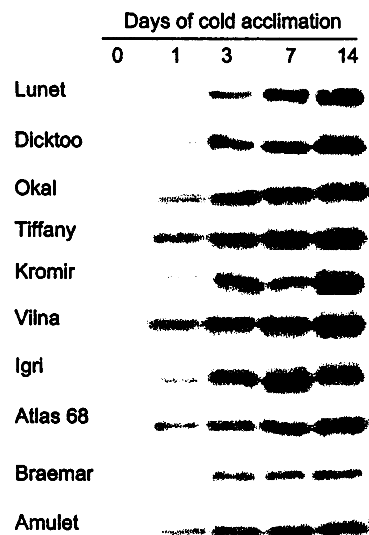

Figure 2. The dynamics of DHN5 accumulation in 10 barley cultivars belonging to different growth habits after 0 (control plants), 1, 3, 7 and 14 d of CA. The cultivars are ordered according to their LT_{50} values (in descending order).

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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

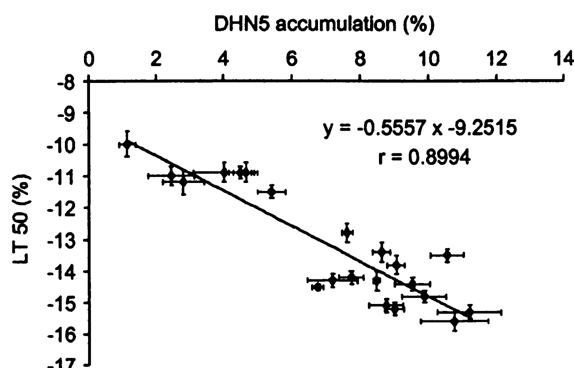

Figure 4. The relationship between DHN5 accumulation (mean values) and FT in 21 barley cultivars after 21 d of CA. Both horizontal bars and vertical bars indicate SE ($n = 4$). The level of DHN5 accumulation is expressed relatively in percent; 100% = total amount of DHN5 in 21 cultivars. Variability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

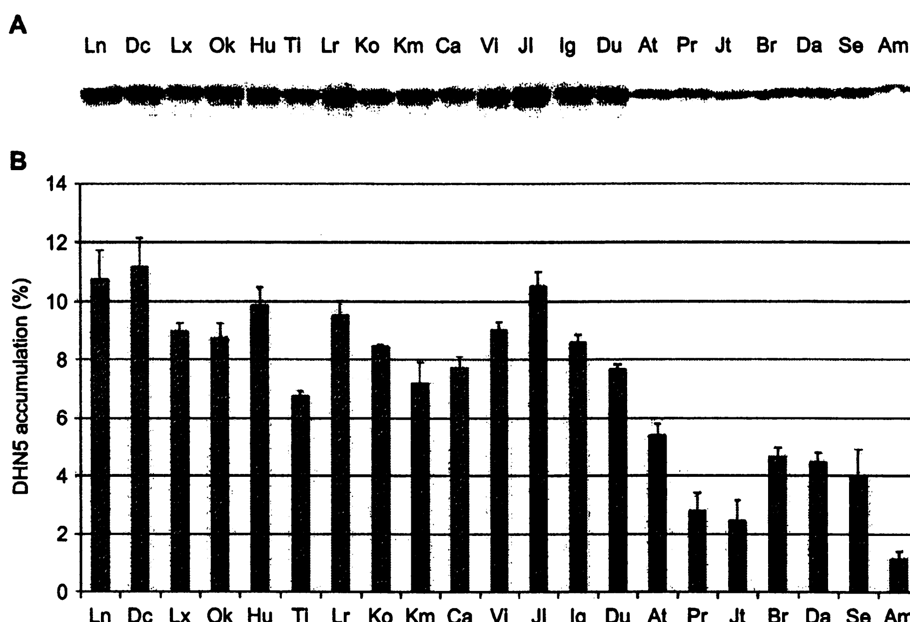

Figure 3. The accumulation of DHN5 in 21 cultivars after 21 d of CA. Protein gel blot (A) and its densitometric analysis (B). The cultivars are ordered according to their LT_{50} values after 21 d of CA (in descending order). The columns represent means obtained from four repetitions, the vertical bars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

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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

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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

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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.

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PAPER 4

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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

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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.

Keywords

Cold acclimation; dehydrin 5; frost tolerance; phenological development; doubled haploids; *Hordeum vulgare*

Introduction

Frost tolerance (FT) is an important ecophysiological adaptation of plants which enables them to survive long periods of low temperatures (cold and frost) during winter. In barley, as well as in many other plants from temperate climate habitats, FT is not a constitutive trait, but it is inducible upon the conditions of low, but above-zero temperatures. This

adaptive process is called cold acclimation (CA) (Sakai and Larcher, 1985; Guy, 1990; Thomashow, 1999) and is associated with *de novo* synthesis of many organic low-molecular compounds as well as proteins. Thus the level of acquired FT can be determined either directly as plant survival after an exposure to frost or indirectly via the accumulation of some of the cold-inducible compounds (Prášil *et al.*, 2007).

During CA, the processes associated with cellular dehydration are induced. The level of endogenous abscisic acid (ABA) rises transiently when compared with optimum growth temperatures. Increased amounts of hydrophilic, low-molecular compounds are also synthesized. These compounds (low-molecular saccharides, polyamines, imminoacid proline, quaternary ammonium compounds called betaines, sugar alcohols, *etc.*), commonly known as compatible solutes, retain a relatively high osmotic potential of the cells and prevent the cells from excessive water loss during freezing. Apart from these low-molecular compounds, specific cold-inducible proteins from the *Cor/Lea* gene superfamily can retain relatively large amounts of water, interacting with other intracellular structures and acting thus as chaperones (preventing other proteins and endomembranaceous structures from unfavorable conformational changes caused by cellular dehydration) (Guy, 1990; Ingram and Bartels, 1996; Thomashow, 1999). Among COR/LEA proteins, some of the LEA II proteins called dehydrins accumulate to relatively high levels upon cold (on the roles of dehydrins upon cold, see *e.g.*, Close, 1997; Allagulova *et al.*, 2003; Rorat, 2006; Kosová *et al.*, 2007).

It has been known for quite a long time that in barley, the major cold-inducible dehydrin protein is DHN5 (Van Zee *et al.*, 1995; Bravo *et al.*, 1999; Choi *et al.*, 1999; Zhu *et al.* 2000). Based on our previous work (Kosová *et al.*, 2008), we can state that the quantitative amount of DHN5 accumulation corresponds with the acquired level of FT after three weeks of CA when significant differences in FT are reached. Therefore, the accumulation of DHN5 protein can be used for estimation of acquired FT level in barley. This situation is analogous to that in common wheat (*Triticum aestivum*), where the cold-inducible dehydrin WCS120 has been proposed a marker of FT (Houde *et al.*, 1992; Vítámvás *et al.*, 2007).

The expression of many cold-inducible *Cor/Lea* genes is regulated by the two major *Frost-resistance* (*Fr*) loci in barley, named *Fr-H1* and *Fr-H2* (Vágújfalvi *et al.*, 2000). Both the *Fr-H1* and the *Fr-H2* loci are located on 5HL in barley; however, the genetic distance between the two loci is quite high (ca 25 cM), so the recombination events between the two loci are possible (Vágújfalvi *et al.*, 2000; Francia *et al.*, 2004). To the *Fr-H2* locus, a cluster of *CBF* genes, which are known from *Arabidopsis thaliana* as important regulators

of *Cor* gene expression (Jaglo-Ottosen *et al.*, 1998), has been mapped (Choi *et al.*, 2002; Francia *et al.*, 2004, 2007; Skinner *et al.*, 2005; Stockinger *et al.*, 2007). The nature of the *Fr-H1* locus remains unknown; however, it becomes evident that this locus cannot be clearly separated from the major vernalization locus, *VRN-H1*, in barley (Francia *et al.*, 2004; Stockinger *et al.*, 2007). The strong effect of the allelic constitution at the *VRN-1/Fr-1* locus (*i.e.*, spring-type *Vrn-1/Fr-1* versus winter-type *vrn-1/Fr-1*) on the acquired FT and the expression of *Cor/Lea* transcripts and corresponding proteins has been repeatedly proven by many researchers (see *e.g.*, Kobayashi *et al.*, 2005; Stockinger *et al.*, 2007).

It is also well-known in *Triticeae* that the capacity to induce FT upon CA is strongly dependent on the plant developmental stage. It has been confirmed by many researchers (*e.g.*, Fowler *et al.*, 1996a,b, 2001; Mahfoozi *et al.*, 2001; Danyluk *et al.*, 2003; Prášil *et al.*, 2004) that the developmental transition from the vegetative stage into the reproductive stage is accompanied by a significant loss in the capacity to induce increased FT level and to accumulate COR/LEA proteins upon cold. Recently, it has become evident that the expression of the product of the major vernalization gene in *Triticeae*, the *VRN-1* gene, is necessary for the transition into the reproductive stage (Shitsukawa *et al.*, 2007). In winter growth habit of *Triticeae*, which is capable of surviving long-term low-temperature treatments, a requirement of a sufficiently long period of low temperatures does exist which prevents the plants from a premature developmental transition into the less frost-tolerant reproductive stage. The requirement of a long-term low-temperature treatment prior to the developmental transition into the reproductive stage is called vernalization (Chouard, 1960). Vernalization results in the down-regulation of the expression of the vernalization gene *VRN-2* which is a repressor of *VRN-1* gene (Yan *et al.*, 2004). Thus, the *VRN-1* gene becomes expressed and the plant proceeds to the reproductive stage (Danyluk *et al.*, 2003; Kane *et al.*, 2005). In spring growth habit of *Triticeae*, which is not able to postpone the developmental transition, vernalization requirement had been lost as a consequence of loss-of-function mutations in the major vernalization gene *VRN-1* that result in irrepressible *Vrn-1* alleles dominant over repressible *vrn-1* alleles (Yan *et al.*, 2003, 2004). Thus, *Triticeae* of the winter growth habit, which possess only recessive *vrn-1* alleles, can retain a relatively high level of acquired FT and high level of COR/LEA proteins under a long-term CA, while the plants of the spring growth habit fail in the maintenance of an enhanced FT and COR protein levels upon longer periods of CA. Recently, Stockinger *et al.* (2007) have proven on a set of recombinant lines between Nure (a frost-tolerant winter barley cultivar) × Tremois (a frost-sensitive spring barley cultivar)

that the allelic constitution at the *VRN-H1/Fr-H1* locus significantly affects the expression of some *CBF* genes located at the *Fr-H2* locus, thus affecting the plant capacity to induce FT under CA. In common wheat, the experiments carried out on winter wheat Norstar, spring wheat Manitou and two near-isogenic lines with interchanged *VRN-A1* locus ('spring Norstar' and 'winter Manitou') have shown that the allelic constitution at the *VRN-A1* locus and the resulting growth habit do strongly affect the maintenance of enhanced FT level and the expression of some *Cor/Lea* transcripts (mRNAs) under a long-term CA (Danyluk *et al.*, 2003; Kane *et al.*, 2005; Ganeshan *et al.*, 2008).

In this work, we have used a set of selected doubled haploid (DH) lines (*i.e.*, homozygous in all alleles including the major vernalization gene *VRN-H1*) between Atlas 68, a spring barley cultivar, and Igri, a winter barley cultivar, in order to analyse the induction, the maintenance, and the subsequent loss of acquired FT level under a long-term CA. Our aim was to compare the dynamics of the development of FT in spring and winter DH lines during a long-term CA with respect to their phenological development. DHN5 protein accumulation was determined during the whole CA treatment in order to test the possibility of the use of DHN5 protein as a potential marker of acquired FT level in barley.

Material and methods

Plant material

For all experiments, the plants of Atlas 68, a spring barley (*Hordeum vulgare* L.) cultivar, and the plants of Igri, a winter barley cultivar with a relatively moderate FT were used (Kosová *et al.*, 2008).

In addition to these two cultivars, a set of DH lines derived from an Atlas 68 × Igri cross was used in experiments described in section 2 of the Results. The selected DH lines exhibited different levels of frost tolerance and a presence (winter DH lines) or an absence (spring DH lines) of vernalization requirement. The DH lines used in the experiment were the following: 07, 020, 027, 034, 045, 051, 057, 071, 082; 3, 13, 36, 59, 63, 67, 68, 81, 89, 94, 111, 113.

Cultivation of plants

The seeds were put on moist filter paper and let germinate under controlled temperature (21 °C). The germinated seeds were then planted into the pots filled with soil and cultivated in growth chambers (Tyler, type T – 16/4, Budapest, Hungary) at 20 °C and 12 h photoperiod

(400 µmol m^{-2} s^{-1}) until the 3-leaf stage. When the plants reached the 3-leaf stage, the temperature was decreased to 3 °C (CA treatment). The tissue of young, but fully expanded leaves of Atlas 68 and Igri plants was collected for protein analysis at 0, 3, 7, 10, 25, 35, 49, 63, 77, 91 and 112 days of CA. In experiments carried out with the DH lines (experiments described in section 2 in Results), the sampling dates were the following: 0, 21, 42, 63 and 84 days of CA.

Determination of vernalization requirement as days to heading

Plants after a certain period of CA treatment were transferred to optimum growth temperature (20 °C) and watered and fertilized regularly. The time necessary to heading was determined as days to heading, *i.e.*, a period of time for which the plants have to be grown at optimum growth temperature after a certain period of CA treatment.

Phenological development of shoot apex

The phenological development of shoot apex was determined on Atlas 68 and Igri plants at 0, 21, 42, 63 and 84 days of CA. After dissection, the apices were evaluated under magnification 10 × 50. The stage of phenological development was determined according to the decimal scale published by Nátrová and Jokeš (1993).

Frost tolerance tests

Frost tolerance tests were carried out in a set of laboratory freezers under controlled temperature conditions. The tests were carried out at a defined freezing temperature for 24 h, both the rates of cooling and thawing were 2 °C h^{-1} (Prášil and Zámečník, 1998).

In experiment described in section 1 of the Results, the direct frost tests were conducted on leaf segments whose damage after the frost test was determined by conductivity measurements and the lethal temperature values when 50 % of a sample die (LT_{50} values) were calculated according to Janáček and Prášil (1991).

In experiment described in section 2 of the Results, the direct frost tests were carried out on the plant segments without leaves and the level of acquired FT was determined from the regeneration of the plant segments after a defined period of growth at optimum temperature conditions (20 °C). The temperatures used in the direct frost test did not vary during the whole CA treatment (sampling dates: 0, 21, 42, 63 and 84 days of CA). The following temperatures were used in the frost test at each sampling date: -10 °C, -12 °C and -14 °C.

Analysis of DHN5 protein content

Plant leaf tissue from young, but fully expanded leaves was frozen in liquid nitrogen and stored at -80 °C until the extraction of proteins soluble upon boiling. 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 fresh weight (FW) of the sample; e.g., to 1 g FW of the sample, 5 mL of the buffer were added. 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 were added. The samples were loaded on 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 (pore diameter 0.45 μm; Bio-Rad). Membranes were blocked in 3 % gelatin in 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 (Close *et al.*, 1993) 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 GAR-AP 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 AP Conjugate Substrate Kit (Bio-Rad) until the reaction was completed (about 5 – 7 min).

The amount of accumulated DHN5 protein on the protein gel blots was analysed densitometrically using Quantity 1D – software (Bio-Rad, version 4.6.2).

Statistical analysis

Statistical analyses were performed using ANOVA tests, multiple comparisons, at Unistat Program (Unistat Ltd.,UK, version 5.1). Significant differences in growth habit, day of CA and growth habit × day of CA interactions were determined as P-values lower than 0.01 .

Results

1/ Days to heading, frost tolerance, DHN5 level and phenological development in Atlas 68 and Igri during a long-term CA (0 – 112 days of CA)

During 112-day CA at 3 °C and 12 h photoperiod, the following physiological characteristics of the CA response of Atlas 68 and Igri were evaluated: the days to heading, the acquired level of FT, the level of accumulation of DHN5 protein and the phenological development of shoot apex (Fig. 1, Table 1).

The number of days to heading did not change significantly during the whole CA treatment (112 days) in Atlas 68, a spring barley cultivar (Fig. 1A). The number of days to heading of Atlas 68 varied around 38 – 43 days during the whole treatment. In Igri, in contrast to Atlas 68, the number of days to heading was strongly dependent on the length of CA treatment. It significantly decreased from 172 days before the CA treatment to around 55 – 60 days at 63 days of CA. After this period, the time to heading did not shorten significantly with the prolongation of the CA treatment. This result indicates that the vernalization requirement of Igri grown at neutral (12 h) photoperiod was around 63 days.

The level of acquired FT increased rapidly in both Atlas 68 and Igri during the first 35 days of CA (Fig. 1B). After that, the acquired FT of Atlas 68 reached its maximum value (LT_{50} around –13 °C) and began decreasing slowly during the later stages of CA. Contrary to Atlas 68, the acquired level of FT in Igri continued rising, although the rate of the FT increase (decrease in LT_{50} values) was much slower in the later stages of CA than in the first 14 – 21 days of CA. Igri reached its maximum acquired FT after ca 70 days of CA (the LT_{50} value around –17 °C). So it can be concluded that Atlas 68 and Igri did not differ only in the absolute values of the maximum acquired FT, but also in the length of CA when the maximum acquired FT was reached.

No DHN5 protein was present in control plants of both cultivars grown at 20 °C, but since the beginning of CA treatment, both Atlas 68 and Igri started accumulating the DHN5 protein (Fig. 1C). In the first 25 days of CA, the amount of accumulated DHN5 protein

rose in both cultivars and even the quantity of accumulated DHN5 protein was quite similar in both cultivars (there were no significant differences in the amount of accumulated DHN5 protein between the two cultivars). At 25 days of CA, the maximum DHN5 accumulation in Atlas 68 was reached. However, after the first 25 days of CA, the amount of accumulated DHN5 protein dropped rapidly in Atlas 68 and remained low until the end of CA treatment in this cultivar. Contrary to Atlas 68, the amount of accumulated DHN5 protein in Igri rose steadily till 63 days of CA when the maximum DHN5 accumulation in this cultivar was reached. The amount of accumulated DHN5 then decreased slightly; however, it remained relatively high also at 77 and 91 days of CA and it eventually dropped at 112 days of CA. At 112 days of CA, the level of DHN5 accumulation in Igri was significantly lower when compared to the previous sampling dates (63, 77 and 91 days). It was also found out that at 112 days of CA, there was no statistically significant difference in the level of DHN5 accumulation between Atlas 68 and Igri. Therefore, it can be concluded that Atlas 68 reached its highest level of DHN5 accumulation much earlier than Igri and the highest level of DHN5 accumulation in Atlas 68 was lower when compared to the maximum level of DHN5 accumulation in Igri. A representative protein gel blot showing the accumulation of DHN5 protein in Atlas 68 and Igri during the CA treatment is given in Fig. 2.

The phenological development of shoot apex in Atlas 68 and Igri, which was evaluated at 0, 21, 42, 63 and 84 days of CA, has clearly shown the different dynamics of development in Atlas 68 and Igri plants under the same CA treatment (Table 1). The transition to the reproductive stage was faster in Atlas 68 (the double-ridge stage in Atlas 68 was reached at 42 days of CA) than in Igri (the double-ridge stage was reached at 63 days of CA). This difference can be explained by the presence of the vernalization requirement in Igri which prevents the winter cultivar from the fast developmental transition and the absence of vernalization in spring cultivar Atlas 68.

2/ Days to heading, frost tolerance and DHN5 accumulation in selected doubled haploid (DH) lines derived from Atlas 68 × Igri cross during a long-term CA (0 – 84 days of CA)

The plants of Atlas 68, Igri and twenty-one DH lines were grown under CA conditions for 0, 21, 42, 63 and 84 days. At these time points, two representative plants from each line were transferred to optimum growth conditions (20 - 21 °C) and the vernalization

requirement was determined as days to heading. At the same time points, the acquired FT level was determined from the regeneration of plant segments after a direct frost test and the level of DHN5 accumulation was determined from densitometric analysis of the protein gel blots (Fig. 3).

It has turned out that ten of the selected DH lines (07, 020, 027, 034, 045, 051, 057, 071, 082, 67) did not exhibit any significant decrease in days to heading with the progress of CA, while in the remaining eleven DH lines (3, 13, 36, 59, 63, 68, 81, 89, 94, 111, 113), the increase in the length of CA significantly reduced the number of days to heading (Fig. 3A). Therefore, it can be concluded that ten of the selected DH lines were of spring growth habit (they do not have vernalization requirement) and the remaining eleven DH lines were of winter growth habit (they have vernalization requirement). (In the following text and in Fig. 3, the term spring DH lines refers to the ten DH lines without vernalization and to the parental cultivar Atlas 68, while the term winter DH lines refers to the eleven DH lines with vernalization and the parental cultivar Igri.)

The development of FT in all DH lines at 0, 21, 42, 63 and 84 days of CA was determined as regeneration of plant segments at optimum growth temperature (20 °C) after a direct frost test (Fig. 3B). It can be concluded that the differences in FT level between spring DH lines and winter DH lines increased with the length of CA (*i.e.*, at 21 days of CA, the differences in FT between spring DH lines and winter DH lines were quite low, and they increased at 63 and 84 days of CA when the FT level in spring DH lines significantly dropped, but the FT level in winter DH lines remained high). The average values of plant survival rate after the frost test at 21, 42, 63 and 84 days of CA have shown that spring and winter DH lines did not differ significantly at 21 days of CA. At 42 days of CA, the survival rates for spring and winter DH lines were 75 % and 92 %, respectively, and a statistically significant difference was found. The winter DH lines were able to maintain high survival rates during the whole CA treatment while the spring DH lines failed in the maintenance of the high survival rates at the later stages of CA treatment.

The relative accumulation of DHN5 protein in selected spring and winter DH lines at 0, 21, 42, 63 and 84 days of CA (the total DHN5 accumulation in Atlas 68, Igri and twenty-one DH lines was set to 100 % at every sampling date) is shown in Fig. 3C. It is obvious that during the first 21 days of CA, the accumulation of DHN5 was induced in all lines (no DHN5 protein was present in control plants grown at 20 °C prior to the CA treatment) and the amount of accumulated DHN5 protein was quite similar in spring and winter DH lines. However, with the progress of CA, the level of accumulated DHN5 rose in the winter DH

lines relative to the spring DH lines. The highest difference in the amount of accumulated DHN5 in the winter DH lines relative to the spring ones was observed at 63 days of CA. At 84 days of CA, the ratio of accumulated DHN5 protein in the winter DH lines relative to the spring DH lines was lower than at 63 days of CA, *i.e.*, the difference in DHN5 accumulation in the winter DH lines with respect to the spring ones was lower at 84 days of CA than it had been at 63 days of CA when the maximum difference in DHN5 accumulation between winter DH lines and spring DH lines was found. This result has been obtained due to a decrease in DHN5 accumulation in the winter DH lines when compared with the situation at 63 days of CA, while the amount of accumulated DHN5 protein in the spring DH lines did not significantly differ from the previous sampling date at 63 days of CA (data not shown). (In Fig. 3C, it looks like if the DHN5 amount in the spring DH lines at 84 days of CA had been higher than at 63 days of CA. However, Fig. 3C shows only relative DHN5 accumulation in spring and winter lines determined separately at each sampling date, so the relative increase in DHN5 in the spring DH lines at 84 days of CA compared to 63 days of CA was a consequence of a significant decrease in DHN5 accumulation in the winter DH lines at 84 days of CA compared to 63 days of CA.) This result obtained on a set of Atlas 68, Igri and twenty-one differently frost tolerant DH lines consisting of ten spring lines and eleven winter lines has confirmed the result obtained on the parental cultivars Atlas 68 and Igri alone since the time (63 days of CA) when the maximum difference in DHN5 accumulation between winter DH lines and spring DH lines was reached was the same as the time when the maximum difference in DHN5 accumulation was observed in Atlas 68 and Igri alone. The dynamics of DHN5 protein accumulation in the spring and winter DH lines during the whole CA treatment was analogous to the dynamics of DHN5 protein accumulation in Atlas 68 and Igri (data not shown). Representative protein gel blots showing the accumulation of DHN5 protein in Atlas 68, Igri and twenty-one DH lines at 21, 42, 63 and 84 days of CA are given in Fig. 4.

Discussion

Many studies have investigated the dynamics of the development of FT with respect to the plant development in barley as well as in wheat and rye under the conditions of CA (*e.g.*, Fowler *et al.*, 1996a,b; Mahfoozi *et al.*, 2001; Prášil *et al.*, 2004). Many of these studies (*e.g.*, Fowler *et al.*, 2001; Danyluk *et al.*, 2003; Kane *et al.*, 2005; Ganeshan *et al.*, 2008) have also found out that the changes in FT associated with the developmental transition

from the vegetative stage into the reproductive stage can be detected as changes in the expression of some *Cor/Lea* genes or their protein products.

As it is known from literature, DHN5 is the most abundant cold-inducible dehydrin in barley (Van Zee *et al.*, 1995; Bravo *et al.*, 1999; Choi *et al.*, 1999; Zhu *et al.*, 2000). Our previous work (Kosová *et al.*, 2008) has shown that the amount of accumulated DHN5 protein corresponds quite well to the acquired level of FT in barley. The dynamics of DHN5 accumulation under CA in barley as well as the CA-induced accumulation of the DHN5 orthologue, the WCS120 protein in common wheat, was studied mostly only in the early stages of CA process (0 – 30 days of CA - see *e.g.*, Van Zee *et al.*, 1995; Bravo *et al.*, 1999; Zhu *et al.*, 2000; Vítámvás *et al.*, 2007; Kosová *et al.*, 2008). In these works, only the increase in DHN5 or WCS120 accumulation was observed (the CA treatment was not long enough to observe the drop in dehydrin accumulation in spring cultivars after a certain period of CA). The only work on dehydrin accumulation during a long-term CA in barley was published by Fowler *et al.* (2001) who evaluated the amount of accumulated cold-inducible dehydrin and LEA III proteins (detected by anti-WCS120, anti-WCOR410 and anti-WCS19 primary antibodies) in Dicktoo, a highly frost-tolerant, but strongly photoperiodically sensitive facultative barley cultivar. The authors have found out that long-day photoperiod (LDs) led to the acceleration of development (indicated by the formation of double-ridge structure in the plant shoot apices) which was accompanied by a decrease in FT and a significant decrease in COR/LEA protein accumulation. In contrast, the plants of the same cultivar developed much more slowly under short-day photoperiod (SDs), they were able to remain in the vegetative stage of development for a longer period of CA and they also retained relatively high FT level and COR/LEA protein level for a much longer period of CA than the same plants under LDs. Thus, the authors concluded that the plant developmental stage has a prominent impact on the acquired FT and COR/LEA protein levels in barley.

Similar results have been obtained in common wheat by Danyluk *et al.* (2003), Kane *et al.* (2005), Ganeshan *et al.* (2008) and others who used the spring cultivar Manitou, winter cultivar Norstar and two reciprocal near-isogenic lines 'winter Manitou' and 'spring Norstar' in their experiments. These authors have found out that the ability to maintain an enhanced level of FT and an enhanced level of transcripts of *Cor* genes under a long-term CA correlated well with the developmental transition into the reproductive stage and the beginning of *VRN-1* gene expression in these lines (*i.e.*, the lines were able to maintain the increased FT and enhanced *Cor* transcript levels until the expression of *VRN-1* gene). The

fact that the ability to maintain the expression of cold-inducible genes upon CA treatment is dependent on the growth habit has been confirmed by Monroy *et al.* (2007) on a winter and a spring wheat cultivar by microarray analysis of cold-inducible transcriptome. The researchers have found out that both cultivars showed the initial burst of cold-inducible gene activity after the beginning of CA; however, the spring cultivar exhibited only a transient expression of cold-inducible genes in the initial phases of CA while the winter one was able to maintain the enhanced expression of cold-inducible genes for a much longer time.

In our experiments, we worked with a set consisting of spring barley cultivar Atlas 68, winter barley cultivar Igri and twenty-one DH lines of both growth habits. The DH lines contain allelic combinations from both parental cultivars in a homozygous state and present a valuable material for the study of the impact of CA treatment on different growth habits. Our experiments confirm the results obtained by the authors mentioned above on the wheat Manitou-Norstar lines and they indicate that analogous relationship between the dynamics of development and the ability to maintain enhanced FT and COR protein levels also exists in barley. Moreover, our results show that at least in the winter cultivar Igri, the developmental transition into the reproductive stage indicated by the double-ridge formation in the shoot apex precedes the drop in FT and DHN5 accumulation. The double-ridge structure in Igri was observed already at 63 days of CA when the maximum acquired FT and the maximum level of DHN5 accumulation were reached. The double-ridge stage indicates the phase of the development which irreversibly points toward flowering, *i.e.*, where is no chance of the return to the vegetative stage at the double-ridge stage (Hay and Ellis, 1998; Prášil *et al.*, 2004). These experimental observations have already been validated on molecular level since Danyluk *et al.* (2003) have found out that the beginning of the expression of the *VRN-1* gene which indicates the developmental transition into the reproductive stage precedes the formation of the double-ridge structure in the shoot apex in wheat Manitou-Norstar lines. These authors have also observed a delay in the drop of FT and *Cor/Lea* gene expression level after the double-ridge stage under CA conditions. Fowler and Limin (2004) found out that the progress in CA treatment after the vegetative/reproductive transition negatively affects the subsequent phenological development (double-ridge formation). Analogously to wheat, it can be expected that the progress in CA treatment after the vegetative/reproductive transition slows down not only the phenological development, but also the FT loss in barley, *i.e.*, the barley plants subjected to a continuous CA treatment proceed in the development and lose their acquired

FT level much more slowly when compared with the same plants transferred to optimum growth temperatures. However, the problem how the rate of phenological development under different growth temperatures affects the maintenance and subsequent loss of FT in winter barley needs to be resolved by further experiments.

At 63 days of CA, Igri reached the double-ridge stage which indicates the irreversibility of the developmental transition into the reproductive stage. After this time point, the relative amount of accumulated DHN5 protein began decreasing in Igri. In contrast, the FT level determined as LT_{50} values in Igri did not significantly differ from the FT level in Igri at 63 days of CA, *i.e.*, no significant decrease in FT level was observed during this interval. The difference in the dynamics of DHN5 and FT (LT_{50}) response to CA in this late stage of CA treatment after the developmental transition in Igri indicates that the level of DHN5 protein accumulation reacts faster (or is more sensitive) on the developmental transition than the FT level does. So it can be hypothesized that the developmental transition into the reproductive stage has a stronger impact on the DHN5 protein level than on the acquired FT level in Igri). This observation has been confirmed on a set consisting of the parental cultivars Atlas 68, Igri and twenty-one DH lines when the plant survival rates and the relative level of DHN5 accumulation in the winter lines at 63 and 84 days of CA were compared (Fig. 3B and C). Similar discrepancy between the FT level and the WCS120 protein level has been observed by Vítámvás and Prášil (2008) in the plants of winter wheat Mironovskaya 808 during a re-acclimation process after the developmental transition into the reproductive stage. This discrepancy can be explained by the known fact that the acquired FT level is a complex trait and the level of dehydrin (or COR/LEA protein) accumulation presents only one part of acquired FT level, *i.e.*, the resulting level of acquired FT is determined not only by the level of COR/LEA protein accumulation, but also by other factors – accumulation of saccharides, low-molecular compatible solutes, proline content, *etc.*. The fact that the level of accumulation of COR/LEA protein does not necessarily correlate with the acquired level of FT has recently been reported by Crosatti *et al.* (2008) on COR14b accumulation in five barley cultivars subjected to CA treatment. However, this phenomenon may present an interesting object of further studies.

Conclusions

We have confirmed that the developmental stage (the growth habit) has a prominent impact on the ability to maintain increased FT and DHN5 level in barley during a long-term CA treatment.

We have found out that in the winter barley cultivar Igri, the developmental transition indicated by the formation of the double-ridge structure at the shoot apex precedes the drop in the acquired FT level and DHN5 protein level. The delay in the drop in FT and DHN5 can generally be explained by a negative effect of a continuous CA treatment on the progress of phenological development after the vegetative/reproductive transition, but more experiments are needed.

A discrepancy between the acquired FT level and DHN5 protein level in the winter barley plants after the developmental transition has been found out. This means that in these plants, the level of DHN5 protein is significantly decreased in comparison with the earlier developmental stages (before the transition into the reproductive stage), but the acquired FT level remains high (similar to the FT level before the developmental transition). This discrepancy can be explained by the fact that the level of COR/LEA proteins is only one part of the acquired FT level, *i.e.*, the decrease in COR/LEA protein level does not necessarily correlate with FT.

Thus we can expect that under continuous CA treatment, the level of DHN5 protein accumulation correlates with the acquired FT only in those barley plants that are in the vegetative stage of development. After the vegetative/reproductive transition, a discrepancy between the dynamics of DHN5 accumulation and the acquired FT was found.

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Table 1

Phenological development of shoot apex in Atlas 68 and Igri plants at 0, 21, 42, 63 and 84 days of cold acclimation (CA). The stage of phenological development was determined according to the scale published in Nátrová and Jokeš (1993). Each value was determined on three different plants. I – vegetative stage of development; II – reproductive stage of development (the double-ridge stage and subsequent stages of development).

Cultivar	**Length of cold acclimation (day)**				
	0	**21**	**42**	**63**	**84**
Atlas 68	I / 13	I / 19	II / 22	II / 27	II / 29
Igri	I / 13	I / 16	I / 19	II / 22	II / 24

Legend to figures

Figure 1

Days to heading (**A**), acquired frost tolerance level determined as LT_{50} values (**B**) and relative accumulation of DHN5 protein (**C**) in Atlas 68 and Igri plants during 112 days of cold acclimation treatment. In (**C**), the sum of DHN5 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.

Figure 2

Protein gel blot showing the dynamics of DHN5 protein accumulation in Atlas 68 and Igri plants during 112 days of cold acclimation.

Figure 3

Days to heading (**A**), percentage of plant survival after the direct frost test (**B**) and relative accumulation of DHN5 protein (**C**) in Atlas 68 and ten spring DH lines (simply described as spring DH lines in the Figure legend) and Igri and eleven winter DH lines (simply described as winter DH lines in the Figure legend) at 0, 21, 42, 63 and 84 days of cold acclimation. All values are the mean values for the spring and the winter DH lines, respectively. In (**C**), the sum of relative DHN5 protein accumulation in Atlas 68, Igri and 21 DH lines equals to 100 % at every individual sampling date (21, 42, 63 and 84 days). Vertical bars represent standard errors (SE). Asterisks (*) indicate the data in which statistically significant differences ($P<0.01$) between spring and winter DH lines were found.

Figure 4

Protein gel blots showing the accumulation of DHN5 protein in Atlas 68 (**At**), Igri (**Ig**) and twenty-one DH lines at 21, 42, 63 and 84 days of cold acclimation (**CA**). **M** - 1D SDS-PAGE pre-stained standards, broad range (Bio-Rad). In the names of the spring DH lines 020, 027, 034, 045, 051, 057 and 071, the symbol '0' is omitted due to the lack of space.

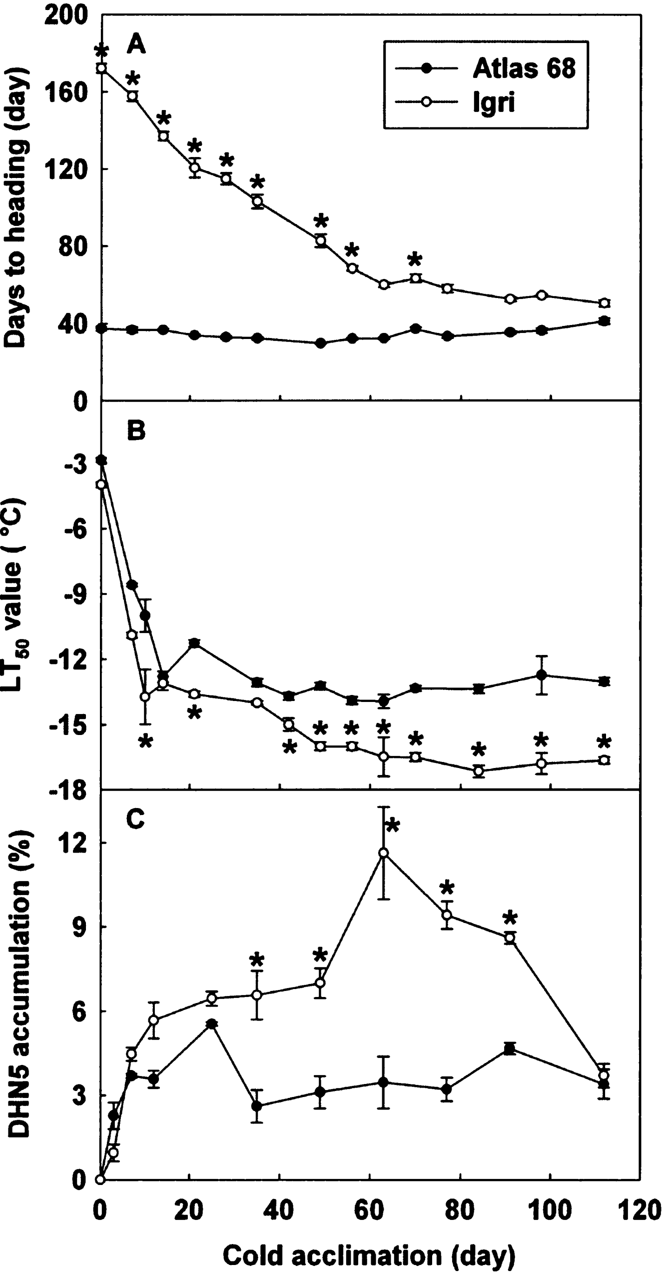

Figure 1

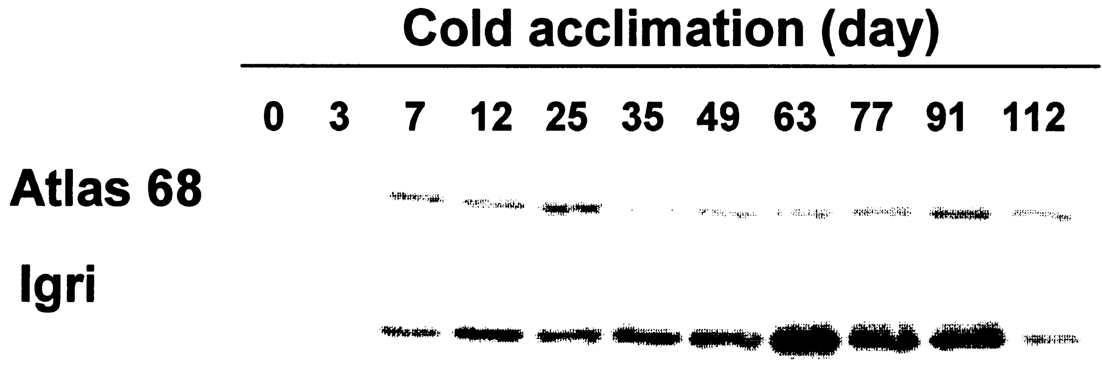

Figure 2

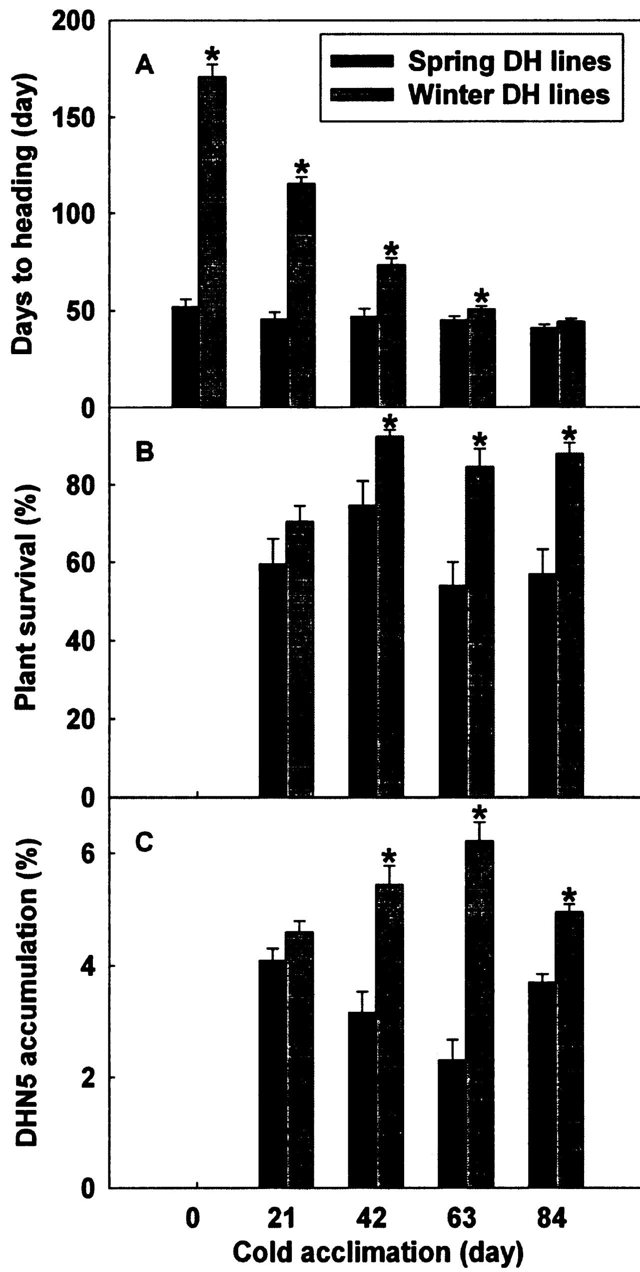

Figure 3

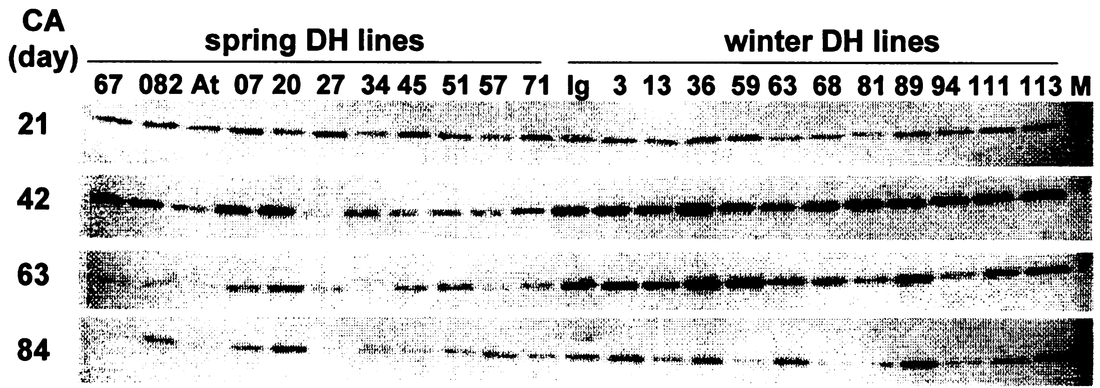

Figure 4