Paper 1

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: LEA D-11 proteins, K-segment, low temperature stress, cold-acclimation, herbaceous and woody dicotyledons, cereals

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; D – dalton; *Dhn* – dehydrin; DRE - dehydration-responsive element; ELIPs - early light-inducible proteins; *Erd* – early response to drought; EST - expressed sequence tag; *Fr* – frost resistance; 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; MW – relative molecular weight in daltons; 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 – sodium dodecyl sulphate; SDS-PAGE – sodium dodecyl sulphate polyacrylamide gel electrophoresis; UV CD – ultra-violet circular dichroism; *Vrn* – vernalization; *Wcor* – wheat cold-regulated; *Wcs* – wheat cold shock;

Wdhn – wheat dehydrin; WT – wild type; 2DE - two dimensional electrophoresis;.2D-DIGE – two dimensional difference gel electrophoresis

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Introduction

Dehydrins, also known as LEA D-11 or LEA II (late embryogenesis abundant) proteins, are proteins whose expressions are induced by various environmental factors which cause dehydration of the cells. Among these factors, cold, frost, drought, enhanced salinity of the substrate and enhanced evaporation are the most notable. Expression of many dehydrins is also induced by increased abscisic acid (ABA) content. The classification of LEA proteins originates from sequence homologies of late embryogenesis 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 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 an α-helical structure may form intramolecular bundles which probably enhance their amphipathic character. These features indicate that dehydrins may function as emulsifiers or chaperones in the cells (Close 1997, Allagulova *et al.* 2003), *i.e.*, that they can interact with cell endomembrane 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 highly ordered structure of the „water envelope“ surrounding these macromolecules

becomes disrupted 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 which is located near the amino terminus and the S-segment which contains multiple serine residues whose function 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 biochemical characteristics of dehydrins, which are: high hydrophility of the proteins, solubility of the proteins in aqueous solutions upon boiling, and their high affinity to detergents such as sodium dodecyl sulphate (SDS) which causes that the apparent molecular weight (MW) of the dehydrins on electrophoretic gels to appear significantly higher than the actual MW of the 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 depending 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

The major abiotic stress factors which cause dehydration of plant cell cytoplasm leading to the decrease of its water potential include drought, increased salinity of the substrate, increased

evaporation, cold and frost stress. 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 maize (*Zea mays*), soybean (*Glycine max*), tomato (*Lycopersicon* sp.), cucumber (*Cucumis sativus*) or cotton (*Gossypium hirsutum*). In contrast, these temperatures induce important biochemical and physiological changes in freezing-tolerant plants such as winter cultivars of rye (*Secale cereale*), wheat (*Triticum aestivum*) or barley (*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 disacharide mellobiose), sugar alcohols (sorbitol, mannitol, pinitol), polyamines (spermine, spermidine, putrescine), quaternary ammonium compounds (glycinebetaine, alaninebetaine) or proline - which decrease the water potential of this cell compartment 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). 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 genetic mechanisms connected with the maintenance of winter hardiness (Fowler *et al.* 2001, Welling *et al.* 2004). Thus, it 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 considered a more rapid process than cold acclimation. Whereas the gaining of a maximum FT by the process of 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).

A given level of FT is one important component of a more complex physiological trait, the plant winter hardiness. 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 stress conditions. 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 response to many environmental stresses including cold is mediated by signalling pathways involving calcium ions. Generally, cytosolic concentrations of calcium ions are extremely low (around 200 nM). Their levels increase by several orders during signalling as a consequence of regulated flow from the 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 calcium-binding activity seems to be specifically induced by cold stress. The authors proposed that ERD14 possessing bound calcium ions may have a function of ionic buffer or carbohydrate chaperone under cold stress similar to calreticulin or calnexin that bind calcium 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 calcium ions and therefore it can be proposed that a calcium-binding activity is a trait shared by acidic dehydrins in *A. thaliana*.

In *Brassica napus* and *Brassica 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 oilseed rape by Deng *et al.* (2005). Other dehydrin genes have also been identified in *Capsella bursa-pastoris* (Fan and Wang 2006).

In the cultivated potato (*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 levels 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 level 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 *Solanum sogarandinum* under cold stress (4 °C).

In freezing-tolerant *Solanum commersonii* and freezing-sensitive *Solanum 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 spinach (*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 alfalfa (*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 alfalfa. Similarly, in cell suspension cultures of *Medicago 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 ripe seeds of *Cicer pinnatifidum*, a wild relative of important tropical and subtropical crop chickpea (*Cicer 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 further characteristics of this protein, 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 the chickpea to a wide variety of environmental stress factors, both abiotic and biotic.

In cowpea (*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 the leaves of young poplar plants of *Populus tremula*×*Populus tremuloides* cv. Muhs1 six prominent bands belonging to proteins of MWs 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 MW 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 peach, a dehydrin PCA60 of Y_2K_9 type with apparent MW 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 cultivar. 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 flower buds of cold tolerant blueberry plants (*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 posttranslational 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 citrus trees, dehydrins were first identified by Cai *et al.* (1995) in a cold-tolerant trifoliate orange (*Poncirus trifoliata*). The two cold-induced dehydrins of KS- type identified in *P. trifoliata* were described as COR11 and COR19. In grapefruit (*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 Satsuma mandarin (*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 orange (*Citrus sinensis*) and grapefruit (*Citrus paradisi*). The levels of their mRNAs increase in chilled fruits after a brief hot water treatment. They are present only in one copy per genome.

In pistachio (*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 silver birch (*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/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 pubescent birch (*Betula 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 fact that dehydrins and other stress-related proteins are associated with the cold acclimation of woody plants was observed by Wisniewski *et al.* (1996) in the bark tissues of eight species – peach (*Prunus persica*) cv. Loring, apple (*Malus domestica*) cv. Golden Delicious, thornless blackberry (*Rubus* sp.) cv. Chester, hybrid poplar (*Populus nigra*), weeping willow (*Salix babylonica*), flowering dogwood (*Cornus florida*), sassafras (*Sassafras albidum*) and black locust (*Robinia pseudo-acacia*). 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 peach 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 apparent MW larger than 106 kD were detected whereas in blackberry and sassafras, several low-MW DHNs with MWs ranging from ca 25 to 30 kD were found. The apparent MWs 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 Scots pine (*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 white spruce (*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). Cultivated barley (*Hordeum vulgare*) 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 wheat, two major groups of dehydrin genes induced by cold have been detected: 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 MWs ranging from 12 to 200 kD: WCS200 (apparent MW 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-MW 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 crown 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).

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 MWs, pIs, 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 apparent MW on immunoblots ranges from 80 to 86 kD (Van Zee *et al.* 1995, Bravo *et al.* 1999) though its MW calculated from its amino acid sequence is only 58.5 kD (Close *et al.* 1995). This discrepancy between actual MW of DHN5 and its apparent MW on SDS gels is also typical for other dehydrin proteins and is caused by their unique amino acid composition (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 cultivars of barley (*e.g.*, Dicktoo) than in freezing-sensitive ones (*e.g.*, 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 deacclimation 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 apparent MWs lower than DHN5 in three barley cultivars after 30 d of cold treatment. The bands were strongly developed especially in the cultivar Frontera. These weaker bands which have remained unidentified have been also observed by us in both the spring cultivar Atlas 68 and winter cultivar 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 MWs 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 low-MW *Dhn* genes and 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*.

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).

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 pathways (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 thaliana* are not able to develop sufficient level of FT comparable with WT upon LT treatment. However, the level 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 thaliana* (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 process 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 by certain developmental processes. It should be noted that only vegetative organs can significantly increase the actual level of 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 cultivar Cheyenne and the freezing-sensitive spring cultivar Chinese Spring it was shown by Limin *et al.* (1997) 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 cultivar 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 cultivar 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 cultivar Norstar and spring wheat cultivar 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 cultivars 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 ºC) more than lower-FT winter or spring cultivars (9 or 4 ºC) (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 cultivar Igri and spring barley cultivar Atlas 68 during 16 weeks of cold acclimation. The spring cultivar 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 Atlas 68, 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 the winter cultivar 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 thaliana* and observed the impact of cold, drought and salt stresses on gene up- or downregulation. The researchers found 53 cold-, 277 drought- and 194 salt-stress 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 thaliana*. 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.

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Tables

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). MWs and pIs were calculated from complete protein sequences using ExPASy (Swiss Prot). MWs 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 no.)	Protein (Protein type) (Protein accession no.)	Number of AA	MW (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 (Y2SK2) (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 (SK3) (CAA62448)	260	29.4	5.12	In root tips.	4/2 °C (day/night).	Wellin *et al.* 1994, 1995
	Lti30/Xero2 (X77613)	LTI30 (K6) (CAA54704)	187	20.1	8.95	In vascular tissues and anthers.	4/2 °C (day/night).	Wellin *et al.* 1994
	ERD14 (D17715)	ERD14 (SK2) (BAA04569)	185	20.8	5.41	In vascular tissues and bordering parenchyma; calcium-binding activity – possibly carbohydrate 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)	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 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	8.09	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

Paper 2

Proteome analysis in plant stress research

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Abstract

The proteomic techniques that allow identification and quantification of stress-related proteins, mapping of dynamics of their expression and posttranslational modifications, represent an important approach in the research of plant stresses. In this review, we show an outline of proteomics methods and their applications in the research of plant resistance to various types of stresses.

Keywords: plant proteomics, mass-spectrometry, two-dimensional gel electrophoresis

Abbreviations: 2-DE, two-dimensional gel electrophoresis; BN-PAGE, blue native-polyacrylamide gel electrophoresis; COFRADIC, combined fractional diagonal chromatography; COR, cold-regulated; DIGE, two-dimensional difference gel electrophoresis; HSP, heat shock protein; ICAT, isotope coded affinity tags; iTRAQ ™, Applied Biosystems trademark name for multiplexed isobaric tagging technology for relative and absolute quantitation; LC, liquid chromatography; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; MS/MS, tandem mass spectrometry; MudPIT, multidimensional protein identification; PAGE, polyacrylamide gel electrophoresis; PR, pathogenesis-related; SELDI, surface-enhanced laser desorption/ ionization; SILAC, stable isotope labeling by amino acids in cell culture; TOF, time-of-flight

Introduction

During their life cycle, plants have to handle a range of biotic and abiotic stresses. Under these stresses, numerous physiological, biochemical and metabolic functions are modified in plants. The research of plant responses to stress on the DNA or RNA level brought us an important insight into defence processes (*e.g.* Fowler and Thomashow, 2002; Gulick et al., 2005; Seki et al., 2001), but it is known that the levels of transcripts and proteins are not strictly correlated as shown in yeast (Gygi et al., 1999; Ideker et al.,

2001). In addition, many proteins are modified by posttranslational modifications such as phosphorylation, glucosylation, ubiquitinylation, sumoylation, and many others (Mann and Jensen, 2003; Schweppe et al., 2003; Canovas et al., 2004) which significantly influence protein functions. Knowledge of the full complement of proteins expressed by the genome of a cell, tissue or organism at a specific time point (i.e. proteome) is necessary to understand the biology of a cell or an organism. The proteome reflects the actual state of the cell or the organism and is an essential bridge between the transcriptome and the metabolome.

Proteins act directly on biochemical processes, and thus must be closer to the phenotype, compared to DNA-based markers. In this context, proteins certainly represent more informative markers compared to DNA markers (Thiellement et al., 2002).

Compared to the analysis of the transcriptome, analysis of the plant proteome in response to abiotic and biotic stresses is still limited, but technical progress has been achieved in the separation of proteins and their identification by mass spectrometry (*e.g.* Canovas et al., 2004; Marra et al., 2006). The methods of proteomics were intimately described in many other reviews (*e.g*. Renaut et al., 2006), so these methods will be only shortly recapitulated. The most frequently used method to investigate differential protein abundance in large-scale proteomics experiments on crude protein mixtures is still two-dimensional gel electrophoresis (2-DE) developed about 30 years ago (O'Farrell, 1975). 2D-DIGE (two-dimensional difference gel electrophoresis) is one of the recent improvements of 2-DE. The basis of this technique is the labelling of an internal standard and two different samples by fluorescent dyes (Cy2, Cy3, Cy5), which are then mixed and separated on one 2-DE gel. Among its multiple advantages, DIGE provides quantitative results and increases the dynamic range of the technique (Tonge et al., 2001; Renaut et al., 2006). Non-denaturing 2-DE, referred to as blue-native PAGE (BN-PAGE) (Schagger and von Jagow, 1991), has been developed to study protein complexes. One of the limitations of 2-DE is the low representation of the genome expression, with 2,000 to 3,000 gene products revealed in the best 2-DE gels instead of the 10,000 to 15,000 genes expressed in the same tissue and at the same developmental stage. Thus, fractionation of the crude extract is becoming a necessity (Thiellement et al., 2002).

After the separation of proteins by 2-DE, selection of spots of interest and their isolation, proteins are digested by trypsin and identified using mass spectrometry (MS)

while searching in public databases using computer software programs. If the genome of the plant has already been sequenced, it is usually sufficient to identify a protein of interest by peptide mass fingerprinting. When such genomic data do not exist, sequencing of the peptide by tandem mass spectrometry (MS/MS) may be required to identify the protein of interest (Hirano et al. 2004).

The separation of a complex protein mixture before protein identification by mass spectrometry (MS) could also be done by gel-free methods. Examples of gel-free methods include multidimensional liquid chromatography (LC) in multidimensional protein identification (MudPIT) (e.g. McDonald et al., 2002), combined fractional diagonal chromatography (COFRADIC) for sorting and identification of methionyl, cysteinyl amino terminal, phosphorylated and glycosylated peptides (Gevaert et al., 2003; Gevaert et al., 2005; Martens et al., 2005; Ghesquiere et al., 2006) or proteome chips (protein-microarrays) for analysis of protein-protein interactions and protein activities (Zhu et al., 2001). Using the technology of the protein-chip coupled with bioinformatics, it is becoming possible to screen any protein sample for putative disease biomarkers from a small sample volume by surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) (Caputo et al., 2003; Elek and Lapis, 2006; Kuramitsu and Nakamura, 2006). Recently, the quantification of proteins in the complex protein sample could be solved by stable isotope methods like SILAC (stable isotope labelling by amino acids in cell culture) where the cells are metabolically labelled by non-radioactive amino acids, which makes their proteomes distinguishable from cells labelled by ‘wild type' amino acids (Ong et al, 2002). Some other quantitative methods, like isotope coded affinity tags (ICAT) (Pan et al., 2005; Stroher et al., 2006) or novel mass tagging strategy (iTRAQ ™ Applied Biosystems) (Jones et al., 2006), label peptides by stable isotope through the chemical modification. Gel-free techniques are more automate and are not so time consuming as gel-based techniques. However, they are not widely used in the plant biology yet. This is due to the fact that most of gel-free techniques require the sequence of the entire genome of the organism have to be available (Renaut et al, 2006).

Abiotic and biotic stress

About 200 original articles focusing on plant proteomics have been published during the last two years, i.e., less than 1% of the global proteomics output (Rossignol et al, 2006). These papers concern the proteome of at least 35 plant species but have concentrated

mainly on *Arabidopsis thaliana* and *Oryza sativa*. In these papers, authors used a proteomic analysis of organs, tissues, cell suspensions, or subcellular fractions for the study of plant development and response to various stresses (Rossignol et al, 2006).

Plants responding to environmental stresses (e.g., pathogens, cold, frost, drought, and heat) produce several protective compounds and proteins such as PR proteins (pathogenesis related), HSPs (heat-shock proteins), AFPs (antifreeze proteins), dehydrins and other proteins.

Here we describe only some examples of using proteomics in the research of abiotic and biotic stress.

Abiotic stress

Primary stresses, such as cold, drought, salinity, heat and chemical pollution, and secondary stresses, such as osmotic and oxidative stress cause cellular damage (Wang *et* al., 2003). During these stresses, analogous sets of genes are activated whose products lead to the re-establishment of homeostasis and participate in the protection and restoration of damaged proteins and membranes. For instance, dehydrins, also known as Lea D-11 or Lea II proteins, are proteins whose expression is induced by various environmental factors which cause dehydration of the cells (Close, 1997; Kosová et al., 2007). Among these factors, cold, frost, drought, enhanced salinity of the substrate and enhanced evaporation are the most notable (e.g., Close *et al.* 1995; Kirch *et al.* 1997; Choi *et al.* 1999; Welling *et al.* 2004). Expression of many dehydrins is also induced by an increased level of ABA (Lang and Palva, 1992; Deng *et al*., 2005).

Vítámvás et al. (2007) distinguished, by image analysis of 2-DE gels of heat-stable proteins, two highly frost tolerant winter wheat cultivars differing in the levels of accumulation of wheat dehydrins (WCS120 proteins). Recent DNA markers for frost tolerance could distinguish only spring and winter cultivars (e.g., Vágújfalvi et al., 2000). Differences in proteins patterns were also observed by gel-free technique (LC-MS/MS) in the heat-stable protein extracts of cold-acclimated and non-acclimated plants of winter wheat Mironovskaya 808 (Fig. 1) (Vítámvás et al., 2007).

2-DE analysis of cold stress response has also been used for the study of subproteome of organelles or specific tissues, for instance, the nucleus of *Arabidopsis* (Bae et al. 2003), *Arabidopsis* leaves (Kawamura and Uemura 2003) and poplar leaves (Renaut et al. 2004), rice anthers (Imin et al. 2004) and mitochondria of *Pisum sativum* (Taylor et al. 2005). Moreover, Amme et al. (2006) used DIGE technique for analysing

the cold response in *Arabidopsis* leaves. In the experiments, using 6ºC as cold treatment, 22 spots with at least 2-fold altered expression were found compared to control (20°C); among them 18 were increased and four were decreased. Three of the 18 proteins were identified as dehydrins. Spot identification was performed by MALDI-TOF and ESI-MS/MS (Amme et al., 2006).

However, DIGE experiments were also used not only for cold research. For example, Ndimba et al. (2005) studied the effects of salinity and hyperosmotic stress on plant cellular proteins extracted from *Arabidopsis thaliana* cell suspension cultures by DIGE. Of a total of 2,949 protein spots detected on the gels, 266 showed significant changes in abundance across five independent experiments after NaCl and sorbitol treatments. Using MALDI-TOF MS, they identified 75 salt and sorbitol responsive spots including H+ transporting ATPases, signal transduction related proteins, transcription/translation related proteins, detoxifying enzymes, amino acid and purine biosynthesis related proteins, proteolytic enzymes, heat-shock proteins, carbohydrate metabolism-associated proteins and proteins with unknown biological functions (Ndimba et al., 2005).

Most of these proteomic studies have confirmed previously published data on cold-inducible proteins obtained from one-dimensional SDS-PAGE or gene expression studies.

Biotic stress

The research of biotic stresses also took advantage of proteomic techniques in previous years. The most frequented technique (and one of the cheapest ones) is the identification of differentially expressed spots on 2-DE gels by mass-spectrometry – as in other fields of plant biology. Marra et al. (2006) used bean plants (*Phaseolus vulgaris* L.), fungal pathogens (*Botrytis cinerea*, *Rhizoctonia solani*) and the antagonistic fungus *Trichoderma atroviride* to analyse the changes in the proteomes caused by multiple-player interactions by this technique. In the bean proteome, some of the spots analysed by peptide mass fingerprint (MALDI-TOF MS) corresponded to PR-proteins (pathogenesis-related) and were less up-regulated by the pathogen alone than by both *Trichoderma* and the pathogen interacting with the plant (Marra et al., 2006). Cooper et al. (2003) demonstrated the identification of a previously unknown virus by the analogous technique coupled with LC-MS/MS.

The study of PR-proteins is also important for food research due to the fact that many plant-derived allergens have been identified as members of PR-protein families 2, 3, 4, 5, 8, 10 and 14 (Hoffmann-Sommergruber, 2002). For instance, Hajós et al. (2004) identified allergens in wheat flour by proteomic approach. Having used the separation of proteins on 2-DE gels and their further identification by MALDI-TOF MS, one chitinase, one inhibitor and one heat shock protein were identified in the water and salt-soluble extract of a wheat cultivar (Hajós et al., 2004).

The first description of the novel mass tagging strategy (iTRAQ ™ Applied Biosystems) applied to plant pathogen interactions was done by Jones et al. (2006) who describe changes in phosphoproteome of *Arabidopsis* during *Pseudomonas syringae* infection. They identified five proteins (e.g., dehydrin, HSP 81, putative p23 co-chaperone) being potentially phosphorylated as a part of plant basal defence response.

Conclusion

Proteomic analysis is a very useful tool for providing complex information about the differences in plant proteome during abiotic and biotic stresses. This information can show us the complexity of the plant response to various environmental stress factors and can enable us to find biomarkers of plant tolerance to stresses, which would be usable by breeders. Moreover, it is becoming possible to identify unknown pathogens, quantify the biomarkers in different cultivars or evaluate the quality of plant products using modern proteomic techniques.

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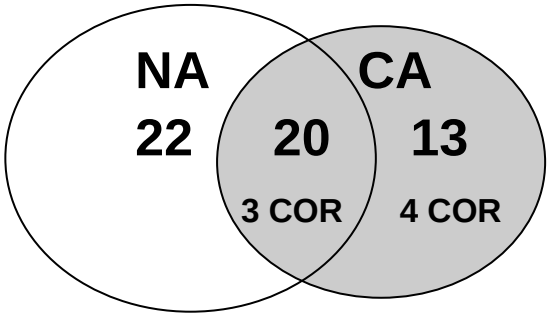
Figures

Figure 1. Results of protein identification by LC-MS/MS on total protein sample of non-acclimated (NA) and cold-acclimated (CA) leaves of cultivar Mironovskaya 808. The numbers represent total identified proteins including COR (cold-regulated) proteins which are presented separately by numbers of identified COR proteins. From Vítámvás et al. (2007).



Paper 3

WCS120 protein family and proteins soluble upon boiling in cold-acclimated winter wheat

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Summary

The amount of proteins soluble upon boiling (especially WCS120 proteins) and the ability to develop frost tolerance (FT) after cold acclimation was studied in two frost-tolerant winter wheat cultivars, Mironovskaya 808 and Bezostaya 1. Protein gel blot analysis, mass spectrometry and image analysis of 2-DE (two-dimensional gel electrophoresis) gels were used to identify and/or quantify the differences in protein patterns before (non-acclimated, NA) and after three weeks of cold acclimation (CA) of the wheats, when frost tolerance increased from -4 °C (LT_{50}, for both cultivars) to -18.6 ºC in Bezostaya 1 and -20.8 ºC in Mironovskaya 808. Only WCS120 protein was visible in NA leaves while all five WCS120 proteins were induced in the CA leaves. Mironovskaya 808 had higher accumulation of three members of WCS120 proteins (WCS120, WCS66 and WCS40) than Bezostaya 1. Mass spectrometry analysis of total sample of proteins soluble upon boiling showed seven COR proteins in the CA samples and only three COR proteins in the NA samples of cultivar Mironovskaya 808. In conclusion, the level of the accumulation of WCS120, WCS66 and WCS40 distinguished our two frost-tolerant winter wheat cultivars. Moreover, the differences of CA and NA samples of cultivar Mironovskaya 808 were shown by LC-MS/MS.

Key words: cold acclimation, COR proteins, frost tolerance, *Triticum aestivum,* winter wheat

Abbreviations

2-DE, two-dimensional gel electrophoresis; CA, cold-acclimated (cultivation at 2 ºC); COR, cold-regulated; BEZ, cultivar Bezostaya 1; FT, frost tolerance; Q, quadrupoles; QM, queries matched; LEA, late embryonic abundant; LC, liquid chromatography; MIR, cultivar Mironovskaya 808; LT50, lethal temperature; MS, mass spectrometry; MS/MS, tandem mass spectrometry; m/z, mass-to-charge ratio; NA, non-acclimated (cultivation at 17 ºC); SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TOF, time-of-flight

Introduction

The ability of plants to survive freezing temperatures, frost tolerance (FT), depends on their genotype and is affected by environment. Higher level of frost tolerance is developed after the exposure of plants to low non-freezing temperatures in the process named cold acclimation (Fowler et al., 1996; Thomashow, 1999).

Cold acclimation involves several biochemical and physiological changes. The research of cold acclimation and frost tolerance has been focused on the characterization of genes that are up- or down-regulated during cold acclimation and are important for the capacity of each genotype to develop FT (Houde et al., 1992a; Galiba et al., 1995; Fowler and Thomashow, 2002; Ohno et al., 2003; Kobayashi et al., 2004). A broad spectrum of cold-regulated genes is represented by the superfamily of *Cor* genes. Proteins encoded by these genes are mostly hydrophilic and soluble upon boiling. They are composed largely of a few amino acids with repeated amino acid sequence motifs, and most of them have amphipathic α-helixes (Thomashow, 1999). Wheat *Cor* genes coding LEA proteins (COR/LEA) were described by many authors, for example: *wcs120* gene family (Houde et al., 1992b; Sarhan et al., 1997), *Wcor410* (Danyluk et al., 1994), *wcs19* (Chauvin et al., 1993; NDong et al., 2002).

The wcs120 gene family encodes a group of highly abundant wheat proteins ranging in size from 12 to 200 kD, namely WCS200 (MW = 200 kD, pI = 6.5), WCS180 (180 kD, 6.5), WCS66 (66 kD, 7.28), WCS120 (50 kD, 7.77), WCS40 (40 kD, 7.30), WCS726 (21 kD, 7.04) and WCS80 (12 kD, 8.05) (Houde et al., 1992a; Ouellet et al., 1993; Sarhan et al., 1997). The expression of the first five major members of the *wcs120* gene family is inducible only by cold treatment, while *wcs726* and *wcs80* genes are inducible also by drought (Sarhan et al., 1997). The WCS120

proteins are rich in glycine and threonine, highly hydrophilic and soluble upon boiling. The WCS120 protein family shares sequence homology with the D11 dehydrin family (Sarhan et al., 1997).

Transcription and/or translation levels of *wcs120* gene alone or together with other *Cor* genes in the plant tissues were used as a proof of induction of *Cor* genes under a cold treatment (Houde et al., 1992b; Danyluk et al., 2003; Shen et al., 2003; Kobayashi et al., 2004). Protein levels have increased rapidly in both spring and winter cultivars following exposure to the cold but the accumulation of WCS120 protein has been higher in winter than in spring cereals (Houde et al., 1992b, Fowler et al., 1996). Houde et al. (1992b) suggest that one of WCS120 proteins, the WCS120 protein, could be used as the molecular marker for frost tolerance in Gramineae. Up to now, the different levels of WCS120 proteins have not been detected between frost tolerant winter wheat cultivars like Mironovskaya 808 and Bezostaya 1. Of winter wheats, Mironovskaya 808 represents a high FT cultivar and Bezostaya 1 a middle FT cultivar (Gusta et al., 2001, Prášilová and Prášil, 2001)

The aim of the present study was to compare two winter wheat cultivars Mironovskaya 808 and Bezostaya 1 with different level of FT analysing their protein patterns. In this work, 2-DE, image analysis, WCS120 antibody were used to study the level of accumulation of the members of WCS120 protein family in both winter cultivars. Furthermore, mass spectrometry analysis of proteins soluble after boiling was used to identify proteins in the NA and CA winter wheat cultivar Mironovskaya 808.

Materials and Methods

Plants

Two winter wheat (*Triticum aestivum* L.) cultivars, Mironovskaya 808 (MIR) and Bezostaya 1 (BEZ) were investigated with regard to their differing ability to develop a frost tolerance. The seeds of these cultivars were kindly received from the breeding company Selgen a.s. Prague.

Growth and cold acclimation

After a seed germination at 18 ºC for 4 days, the seedlings were grown in soil at a constant temperature of 17 ºC under the conditions of a 12 h-photoperiod and an irradiance of 400 μmol m^{-2} s^{-1} supplied by a combination of vapour lamps and high

intensity discharge lamps (LU/400/T/40, Tungsram, Hungary) in a growth cabinet (Tyler, Hungary) (Prášil et al. 2004). After two weeks of growth at 17 ºC, the plants at three-leaf stage were exposed to a cold acclimation at 2 °C with the same photoperiod, for three weeks.

Frost tolerance

The level of frost tolerance was determined by direct freezing of plants in freezing boxes (MHM/52, MIRKOZ, Hungary). Plants taken from the soil were divided into bundles of 10 units and exposed to -4 °C for 20 hours, followed by 5 different freezing temperatures in separate freezing boxes for 24 hours. Temperatures in the boxes differed by 2 °C and were chosen according to the predicted frost tolerance of the plants. The rate of cooling and thawing was 2 °C h^{-1}. After thawing, the plants were cut at 2.5 cm from the crown, the roots were submerged in a dish filled with fresh water and the plants were placed in a greenhouse at 20 °C. After 5 to 6 days, the number of living and regenerating plants was determined for each freezing treatment. The lethal temperature LT_{50} (i.e. the temperature at which 50 % of the plants were killed) was calculated according to the model of Janáček and Prášil (1991).

Protein extraction

Proteins soluble upon boiling were extracted with TRIS buffer (0.1 M TRIS-HCl, pH 8.0 containing "cømplete EDTA-free Protease Inhibitor Cocktail Tablets" (Roche)) from frozen plant leaves as described by Houde et al. (1995), with some modifications. The supernatant after 20,000g-centrifugation was boiled for 15 min and then centrifuged again for 20 min, at the same speed, to eliminate insoluble proteins. COR/LEA proteins are stable upon boiling and therefore the boiling step was used to enrich these proteins in the sample. The soluble proteins were precipitated with 5 volumes of acetone and collected by centrifugation at 20,000 g for 30 min. The pellet was dissolved in sample buffer for SDS-PAGE electrophoresis (Laemmli, 1970) or in rehydration buffer for isoelectric focusing (Bio-Rad). The protein concentration was measured according to Bradford (1976).

Protein analysis

Proteins separated by 2-DE (O'Farrell, 1975) were visualized by Coomassie blue or silver staining. Isoelectric focusing was run on ReadyStrip IPG strips (pH 3-10) in

PROTEAN IEF cell (Bio-Rad) according to the manufacturer's Instruction Manual until 35,000 Vh were reached. The rehydration buffer contained 9.8 M urea and 4 % CHAPS. The focused proteins were then separated in the second dimension by 10 % SDS-PAGE (Laemmli, 1970).

WCS120 proteins were identified on the basis of molecular weight (MW) and isoelectric point (pI) (Houde et al., 1995). "2-D SDS-PAGE Standards" (Bio-Rad) was used for the calibration of MW and pI.

The separated proteins were also identified by the protein gel blot analysis using the WCS120 polyclonal antibody (Houde et al., 1992a). The proteins were electrophoretically transferred to nitrocellulose (0.45 μm, Pharmacia Biotech). After blocking with BSA (3 %) in TBS, the membrane was incubated with a 1:20,000 dilution of WCS120 antibody. After washing with TBS containing 0.05 % Tween-20 and 0.2 % Triton X-100 (Sigma), the secondary antibody (GAR-AP conjugate, Bio-Rad) was applied at 1:3,000 dilution. The complex of proteins and antibodies was visualized by BCIP/NBT staining (Bio-Rad). SDS-PAGE Standards, broad range, (Bio-Rad) were used for the estimation of molecular weight (MW).

Image analysis of 2-DE gels

The image analysis was used to quantify the density of the protein spots on the 2-DE gels. All images have been captured by ColorPage-SP2 (Genius) to the image analysis system LUCIA G v.4.80 (Laboratory Imaging Prague, Czech Republic) equipped with Dual Pentium MMX and Matrox Magic frame grabber. The background of gel was decreased by the function Intensity Transformation on 95 % to 97 % of maximal brightness (255). The spots were defined as the areas of minimally 10 % lower brightness than the brightness of the background. The value of Integral Density was measured on these automatically defined spots. The calibration of density was adjusted as 0 = maximum of the brightness and 10 = black.

Mass-spectrometry / nano-LC-MS/MS

Mass spectrometry was used to identify proteins in the total protein samples after acetone precipitation and centrifugation (as described in Protein extraction) and to determine proteins from the bands chosen from Coomasie stained 2-DE gels.

The protein pellets were dissolved in 50 μL of 0.1 M NH_4HCO_3/10 % acetonitrile. Three μg of trypsin (sequencing grade, Roche) were added and the samples

were incubated at 37 °C overnight. The bands from stained SDS-gels were cut out, washed and treated with trypsin, according to Shevchenko et al. (1996). After the digestion, the peptides were extracted by shaking for 3 h with 100 µL of 5 % formic acid. All tryptic peptide samples were dried and re-dissolved in 50 µL of 0.1 % trifluoroacetic acid/5 % acetonitrile.

LC-MS/MS was used to analyse the tryptic peptides. The analyses were performed on a Q-TOF Ultima Global (Micromass, Manchester, UK) at Risoe National Laboratory (Roskilde, Denmark). A nano-LC system (Famos, Switchos, Ultimate, LC-Packings, Amsterdam, The Netherlands) equipped with RP-columns was used and interfaced directly to the Q-TOF Ultima Global instrument using an electrospray source. The analytical nano-column (75 µm ID x 15 cm) was (self)-packed with Zorbax 300SB-C18, 3.5µm (Agilent Technologies, Palo Alto, CA, U.S.A) and a trap column (300 µm x 5 mm) packed with 5 µm C18, 100Å, PepMap™ (LC Packings) was used. After binding of the peptides to the trap column, the column was washed with 0.1 % trifluoroacetic acid, 2 % acetonitrile and switched on-line with the analytical nano-column. The peptides were eluted and separated by a 92 min gradient of 5-50 % acetonitrile in 1 % acetic acid, 1 % formic acid at a flow rate of 150 nL min^{-1}. The mass spectrometer was run in data-dependent mode (four most abundant ions in each cycle, peak threshold 40 counts s^{-1}, 0.5 s MS m/z 500-2000 and maximum 4 s MS/MS m/z 50-2000, continuum mode, 180 s dynamic exclusion).

Data reduction was performed using MassLynx4.0/ProteinLynx software (peptide filter QA threshold: 100; background subtract: polynomial order 2, below curve 40 %; smooth: 4/1, Savitzky Golay, center: 4 channels, 80 % centroid), and the resulting MS/MS data sets were used to search the NCBInr database using the Mascot search engine (Matrix Science Ltd., London, UK). The sub-database of green plants was searched with a tolerance of 200 ppm (parent ion) and 200 mmu (MS2) and oxidation of methionine as variable modification.

Statistical evaluation was carried out on the basis of a multiple range test (LSD at the 5 % level) of averages calculated from 3 repetitions (Unistat version 5.1, Unistat Ltd., London, UK).

Results

Two winter wheat cultivars Mironovskaya 808 (MIR) and Bezostaya 1 (BEZ) showed similar low frost tolerance ($LT_{50}0$ about -4 ºC) when cultivated at non-acclimated conditions 17 ºC (NA). After 3 weeks of cold acclimation (CA) winter wheat MIR reached LT_{50} = -20.8 ºC and BEZ LT_{50} = -18.6 ºC (Tab. 1).

In relation to increased frost tolerance, water content in leaves decreased after cold acclimation (Tab. 1).

Quantitative analyses of proteins present in the soluble fraction after boiling were related to fresh weight and dry weight (Tab.1). Concentration of proteins was identical in NA leaves of all analysed cultivars. The level of proteins expressed per fresh weight significantly increased in winter cultivars after 3 weeks of cold acclimation but both cultivars had almost the same level of proteins soluble upon boiling. Correspondingly, the level of proteins expressed per dry weight significantly decreased in winter cultivars after 3 weeks of cold acclimation but also without any significant differences between both CA cultivars.

Qualitative and quantitative differences in pattern of soluble proteins stable upon boiling between cold-acclimated and non-acclimated leaf tissues of both wheat cultivars were observed on protein gel blots of 1-DE gels (Fig. 1) and 2-DE gels (Fig. 2). Protein gel blot analysis showed different levels of WCS120 proteins in the NA and CA leaves. NA leaves had all WCS120 proteins invisible except a very low level of WCS120 protein. In the CA leaves of both winter cultivars all five main WCS120 proteins were visible but they had no significantly different levels on 1-DE protein gel blots.

Five intensively silver-stained spots appeared after cold acclimation on 2-DE gels: MW 200 kD- pI 6.5, MW 180 kD- pI 6.5, MW 66 kD- pI 7.28, MW 50 kD - pI 7.77, MW 40 kD - pI 7.30. All of them have been identified as WCS120 proteins, according to their MW and pI value on 2-DE gels (Fig. 2) and using polyclonal WCS120 antibody on W-blotted membranes (not shown). Furthermore, two of them were also confirmed by mass spectrometry (WCS66 and WCS120, see Tab. 2). Three different methods confirmed the expression of cold acclimation-induced WCS120 proteins.

Level of WCS120 proteins expression in analysed cultivars was so distinct on 2-DE gels that image analysis was used to quantify particular WCS120 proteins. According to the sum of all spot densities, the lowest level of WCS120 proteins

accumulation was in BEZ after cold acclimation. Individual WCS120 protein density demonstrated the highest accumulation of WCS66 protein that was almost 1 times higher in MIR than in BEZ. The average values of the density of proteins WCS180 and WCS200 showed no significant differences between both winter cultivars (Tab. 3). Integral density of image analyses revealed the presence of very small amounts of WCS120 proteins in both winter cultivars even in NA samples (Tab. 3).

LC-MS/MS was used to further characterize NA and CA proteins soluble upon boiling in winter wheat MIR. In this way, 33 proteins were identified in CA sample, while 42 proteins were identified in NA sample (see Fig. 3 and Tab. 4). The majority of the identified proteins fell into 3 categories: COR proteins, proteins of photosynthetic apparatus and carbon fixation (photosystem II oxygen-evolving complex protein 1, RuBisCO), and proteins related to translation (RNA binding proteins, ribosomal proteins, ribonucleoproteins (GRP1, cp31AHv, Ps16, product of gene *blt801*). Proteins of the second and the third category were present in both samples. Only three COR proteins appeared in both samples (COR14a, WCOR719, cold-responsive LEA/RAB-related COR protein), while another four COR proteins were specific for CA samples (WCS19, WCOR726, WCOR615 and fragment of WCS200). Moreover, these three COR proteins found in the NA plants had a much lower number of matched peptides and also lower individual scores (2/128, 1/57, 1/55) than in the CA plants (5/251, 3/228, 5/289).

In addition to the above mentioned protein groups, other proteins were detected in samples of MIR. Some of them were found only in CA (like cystein proteinase inhibitor) or in NA samples (like 14-3-3 proteins: GF14 mu, GRF15, 14-3-3 protein).

Discussion

The tolerance of plants to cold and frost is a quantitative inherited trait that involves a wide array of changes, including appearance and accumulation of cold-regulated proteins (COR/LEA). The *Cor/Lea* gene superfamily is a well known group of cold-inducible genes with a positive correlation between their level of expression and the level of freezing tolerance in cereals (Sarhan et al., 1997; Vágújfalvi et al., 2000; Ohno et al., 2001; Kobayashi et al., 2004; Kobayashi et al., 2005). Therefore, the members of this superfamily are possible markers for cold tolerance. However, they were usually used to distinguish spring from winter cultivars.

Our research was focused on the expression of these genes at the protein level in two winter wheat cultivars (Mironovskaya 808 and Bezostaya 1) with high but different level of frost tolerance. Here, we compared NA with three-week CA plants because this time of cold acclimation at regulated conditions was shown to be necessary to reach the high FT in wheat (Fowler et al., 1996; Prášil et al., 2004). After the cold acclimation, Mironovskaya 808 was more frost tolerant than Bezostaya 1, which is coincident with published data (Sutka, 1981; Gusta et al., 2001; Prášilová and Prášil, 2001).

The decrease of water content during a cold acclimation is related to an accumulation of many compounds typical for a cold treatment (Levitt, 1980). The higher concentration of proteins soluble upon boiling (CP) in the CA leaves compared to NA leaves was found in our experiments when it was expressed per fresh weight (FW). However, the opposite result in CP was obtained after a recalculation per dry weight (DW) (Tab. 1). It was shown that major changes in water content were not due to a loss of water but due to a high accumulation of the dry matter (mainly non-protein components like sugars) in the mature leaf during a cold acclimation (Prášil et al., 2001). Therefore, the expression of the concentration of soluble proteins per fresh weight might represent the more correct determination of the protein ratio between NA and CA plant.

The differences in the protein patterns between boiled samples of CA and NA cultivars were recognizable on protein gel blots and 2-DE gels (Fig. 1, 2). All five major WCS120 proteins classified by Sarhan et al. (1997) were visible and identified by both methods in CA plants contrary to NA plants (cultivated at 17 ºC), where only a low level of WCS120 protein was detectable in winter cultivars (Tab. 3). Kobayashi et al. (2004) did not detect any level of *wcs120* mRNA at 25 ºC in NA winter (Mironovskaya 808) cultivar while Fowler et al. (1996) observed very low levels of *wcs120* mRNA at 17 ºC in NA wheats, i.e. at the same temperature which we used for cultivation of plants before acclimation. It could be explained by a threshold temperature below 25 ºC but higher than 17 ºC for the induction of *wcs120* genes. Different threshold temperature was demonstrated by Crosatti et al. (1995; 1996) for *Cor14a* and *Cor14b* in barley and by Vágújfalvi et al. (2000; 2003) for a homologous gene of *Cor14b* in wheat. It was not yet studied if different WCS120 proteins have different threshold temperatures in various cultivars.

Several studies, e.g. Houde et al. (1992b), Fowler et al. (1996), Ohno et al. (2001), NDong et al. (2002), Danyluk et al. (2003), Takumi et al. (2003) and Kobayashi

et al. (2004), showed higher level of expression of *Cor* genes in winter wheat (frost tolerant) than spring wheat (frost sensitive). The research based on protein gel blot analysis of some wheat COR proteins, e.g. WCS120 proteins (Fowler et al., 1996), WCS19 (NDong et al., 2002) or WCOR14, 15 and WDHN13 (Kobayashi et al., 2004) distinguished spring and winter wheat cultivars by protein accumulation. Recently, Gulick et al. (2005) have found 65 genes regulated differently between the cold-acclimated spring cultivar Genlea and winter cultivar Norstar using cDNA microarray. However, in the research on this field there have not been studied or shown any differences in protein accumulation between winter wheat cultivars with different FT. In our studies, we were able to find differences in the quantity of WCS120 proteins on the 2-DE gels (Tab. 3) between two cold-acclimated winter cultivars. Moreover, some of the WCS120 proteins showed good correlation between their density on the 2-DE gels (Tab. 3) and the cultivar's FT (Tab. 1). The sum of the WCS120 proteins was also significantly higher in MIR than in BEZ. The highest difference in protein accumulation was observed in the accumulation of WCS40 protein. Regrettably, the spot of WCS40 had the lowest density in all 2-DE gels of CA plants which limits the use of this protein for determination of differences in FT of winter wheat cultivars. Further, we found no differences between CA winter wheats in the accumulation of WCS180 and WCS200 proteins. On the other hand, the FT of CA wheat cultivars MIR and BEZ could be very clearly distinguished based on the accumulation of WCS120 and WCS66 proteins.

The use of LC-MS/MS made it possible to identify a number of proteins in the total sample of proteins soluble upon boiling (Fig. 3, Tab. 4) and to obtain a more detailed insight into the changes of protein patterns after cold acclimation of cultivar MIR.

We found a higher number of identified COR proteins in the CA samples (7 proteins) than in the NA samples (3 proteins) (Fig. 3). The number of peptides of a specific protein and also the score could depend on the amount of the protein in the sample (Tab. 4). Consequently, the observed peptide numbers and scores could indicate a lower abundance of these three COR proteins in the NA samples (Tab. 4). Members of WCS120 proteins (WCOR726, WCS200) appeared only in CA samples. WCS19 and COR14a (WCOR14) belong to 3 LEA chloroplastic proteins and their accumulation is stimulated by light and cold temperature (Chauvin et al., 1993; NDong et al., 2002). WCOR719 is an actin-binding protein (Danyluk et al., 1996). Cold-responsive

LEA/RAB-related COR protein is responsive to the abscisic acid pathway (Tsuda et al., 2000). WCOR615 shares sequence homology with cold-responsive LEA/RAB-related COR protein and therefore probably belongs to the abscisic acid-inducible proteins.

The presence of 22 proteins identified only in NA plants (like 14-3-3 homologues) and 13 proteins identified only in CA plants (like cystein proteinase inhibitor) indicate that NA and CA samples could be characterized also by other proteins than COR proteins.

The mass spectrometry of total protein sample showed a high number of chloroplastic proteins (e.g. COR14a). This might be caused by a higher abundance of these proteins compared to non-chloroplastic proteins in the leaves. However, most of the observed proteins or transcripts of genes encoding these proteins were found by others in plants after cold treatment. For example, Gulick et al., (2005), found a moderately increased level of transcript of RuBisCO large subunit, a moderately decreased level of transcript of RuBisCO small subunit and a moderately decreased level of transcript of plastid ribosomal protein CL9 in CA (36 days) winter wheat Norstar compared to NA control. Unfortunately, LC-MS/MS of total protein sample under our conditions showed qualitative, not quantitative, differences in proteins and therefore it is almost impossible to compare our results directly with quantitative transcriptomic studies. Only large differences in the protein content (e.g. COR proteins) between CA and NA tissues could be determined based on our results (Tab. 4). The proteins identified as proteins of photosynthetic apparatus and carbon fixation, and proteins related to a translation were found in all samples. However, some of these proteins identified as the homologous proteins (like some of RuBisCO proteins) were found only in the CA or only in the NA plants. This result indicates that different protein isoforms are produced under the different conditions. Therefore, the protein isoforms found only in the CA samples could be associated with the response of plants to cold. Our results show that LC-MS/MS analysis is a useful tool for characterizing differences in protein level in plants with different level of frost tolerance.

In conclusion, it was possible to distinguish two winter wheat cultivars, differing in the degree of frost tolerance, by the accumulation of soluble proteins extracted from CA leaves, namely WCS120 and WCS66. A low level of WCS120 protein was possible to detect at 17 ºC indicating that the threshold temperature for induction of this protein is high for the winter wheat cultivars. Furthermore, the fact that cold-acclimated and non-acclimated samples of the cultivar Mironovskaya 808

were different by LC-MS/MS indicated the possibility to distinguish the plants with different frost tolerance also by this method.

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Tables

Table 1. Lethal temperature (LT_{50}), water content (WC, 100*(g FW-g DW)/g FW), concentration of proteins soluble upon boiling expressed for fresh weight (CP – mg protein/g FW) in leaves of non-acclimated and cold-acclimated wheat cultivars Bezostaya 1 (BEZ) and Mironovskaya 808 (MIR). Values denoted with the same letter are not significantly different between MIR and BEZ within the same treatment (NA or CA).

	Non-acclimated			Cold-acclimated		
cv.	LT_{50} (ºC)	WC (%)	CP (mg)	LT_{50} (ºC)	WC (%)	CP (mg)
BEZ	-4.2a	88a	4.3a	-18.6a	74a	5.3a
MIR	-4.4a	88a	4.2a	-20.8b	74a	5.3a

Table 2. Mass spectrometry (LC-MS/MS) identification of two WCS120 proteins from 2-DE gels of cold acclimated winter wheat Bezostaya 1 (shown in Fig. 2 as WCS120 and WCS66). Individual scores > 34 indicate identity or extensive homology ($p<0.05$).

Protein	GIs	Mass	Score	Queries matched
COLD SHOCK PROTEIN CS66/ WCS66	gi|1169107	46769	47	2
COR39 protein – wheat/ WCS120	gi|7489666	38796	80	3

Table 3. Density of WCS120 protein spots obtained from image analysis of 2-DE gels of non-acclimated and cold-acclimated cultivars Bezostaya 1 and Mironovskaya 808. The given values represent the integral density of spots after subtraction of background density of the 2-DE gels. ND – non-detected. Values denoted with the same letter are not significantly different between MIR and BEZ within the same protein and treatment.

	Integral Density			
	Non-acclimated		Cold-acclimated	
protein	BEZ	MIR	BEZ	MIR
WCS 120	5.0a	7.8a	1220.0a	1734.5b
WCS 66	ND	ND	384.0a	692.7b
WCS 40	ND	ND	79.8a	102.1a
WCS 200	ND	ND	244,0a	288.7a
WCS 180	ND	ND	476.7a	412.5a
sum	5.0a	7.8a	2404.5a	3231.5b

Table 4. Identification of proteins by LC-MS/MS in a total protein sample from non-acclimated (NA) and cold-acclimated (CA) plants of winter wheat Mironovskaya 808. COR proteins are highlighted. Individual scores > 34 indicate identity or extensive homology ($p<0.05$). QM-`queries matched`, ND – non-detected.

Protein	Accession N.	Mass	Score (NA)	QM (NA)	Score (CA)	QM (CA)
nucleic acid-binding protein - maize	S23780	33097	356	5	140	3
Oxygen-evolving enhancer protein 2	Q00434	27253	353	7	226	3
cp31AHv protein - barley	T05725	31903	326	7	250	7
nucleic acid-binding protein - barley	T05727	30662	279	4	256	4
photosystem II oxygen-evolving complex protein 1 - rice (strain Nihonbare)	A38889	26489	265	5	345	4
Ps16 protein - wheat	T06232	31829	230	7	257	5
ribulose 1,5-bisphosphate carboxylase activase isoform 1 [Hordeum vulgare subsp. vulgare]	AAA63163	47113	227	4	230	3
Oxygen-evolving enhancer protein 1	O49079	34848	135	4	152	3
cold-responsive protein COR14a -wheat	AAF17098	13537	128	2	251	5
ribosomal protein L12.1 precursor, chloroplast - rye	S30199	18105	108	1	110	1
glycine-rich RNA-binding protein, low-temperature-responsive – barley (encoded by gene blt801)	S71453	15916	102	2	103	3
Plastocyanin, chloroplast precursor	P20423	15567	61	2	95	2
Thioredoxin M-type, chloroplast precursor (TRX-M)	Q41864	18061	61	1	36	1
RNA binding protein - barley	S53050	16789	58	2	66	2
glycine-rich RNA-binding protein GRP1 - wheat	S71779	16245	58	2	66	2
WCOR719	AAC49404	15755	57	1	228	3
cold-responsive LEA/RAB-related COR protein [Triticum aestivum]	AAF68628	17150	55	1	289	5
RuBisCO subunit binding-protein beta subunit (60 kD chaperonin beta subunit) (CPN-60 beta)	Q43831	53379	53	1	74	2
probable superoxide dismutase (EC 1.15.1.1) (Cu-Zn) precursor, chloroplast - wheat	T06229	20310	51	2	220	4
ribulose-1,5-bisphosphate carboxylase/oxygenase [Lycopodiella cernua]	CAC22264	ND	35	1	40	1
ribulose 1,5-bisphosphate carboxylase/oxygenase large chain [Triticum aestivum]	NP_114267	52817	126	3	ND	ND
Phosphoglycerate kinase, chloroplast precursor	P12782	49809	98	2	ND	ND
ribulosebiphosphate carboxylase [Peridictyon sanctum]	CAA90006	52729	89	3	ND	ND
Oxygen-evolving enhancer protein 3-2	Q41806	22829	76	2	ND	ND
calmodulin [Lycopersicon esculentum]	CAA75056	13307	61	1	ND	ND
Calmodulin	P04464	ND	61	1	ND	ND
ribulose-biphosphate carboxylase large subunit [Blandfordia punicea]	CAA98038	51980	61	2	ND	ND
ribulose 1,5-bisphosphate carboxylase large subunit [Stenomesson variegatum]	AAF97673	48276	58	2	ND	ND
ribulose 1,5-bisphosphate carboxylase large subunit [Cartonema philydroides]	AAD02089	ND	53	1	ND	ND
ribulose-1,5-bisphosphate carboxylase large subunit [Peliosanthes campanulata]	BAA83125	51841	53	2	ND	ND
ribulose-1,5-bisphosphate carboxylase/oxygenase [Tremulina tremula]	AAD50115	49394	53	2	ND	ND

GF14 mu [Arabidopsis thaliana]	AAB49334	29475	50	2	ND	ND
Acyl carrier protein I, chloroplast precursor	P02902	15964	48	1	ND	ND
rpS28 [Hordeum vulgare subsp. vulgare]	CAA04565	7481	44	1	ND	ND
14-3-3 protein [Vigna angularis]	BAB47118	28043	43	3	ND	ND
31 kDa ribonucleoprotein, chloroplast (RNA-binding protein cp31), putative [Arabidopsis thaliana]	NP_199836	ND	42	2	ND	ND
14-3-3 protein (grf15), putative [Arabidopsis thaliana]	NP_565347	ND	42	1	ND	ND
RuBisCO large subunit [Hypecoum imberbe]	AAB88833	ND	39	1	ND	ND
putative methyl-binding domain protein MBD105 [Zea mays]	AAK40307	43684	37	1	ND	ND
putative cytochrome P-450 like protein [Oryza sativa (japonica cultivar-group)]	NP_919986	ND	36	3	ND	ND
ribulose-1,5-bisphosphate carboxylase, large subunit [Mitchella repens]	CAA93017	51608	35	2	ND	ND
ribulose-1,5-bisphosphate carboxylase, large subunit [Plocama pendula]	CAA93028	52159	35	2	ND	ND
oxygen evolving enhancer protein 1 precursor [Bruguiera gymnorrhiza]	BAA96365	35112	ND	ND	247	3
cold acclimation protein WCOR615 - wheat	T06812	17773	ND	ND	190	4
leaf-specific protein Wcs19 - wheat	Q06540	19690	ND	ND	175	3
ESTs AU070372(S13446),AU075541(S0353)	BAA82377	23165	ND	ND	129	2
ribulose bisphosphate carboxylase activase B [Triticum aestivum]	AAF71272	47784	ND	ND	127	2
ES2A protein - barley	Q43478	18371	ND	ND	96	2
cysteine proteinase inhibitor [Triticum aestivum]	BAB18768	13802	ND	ND	51	1
dehydrin-/LEA group 2-like protein [Lophopyrum elongatum]	AAC05923	12755	ND	ND	47	1
probable dehydrin WCOR726 - wheat	T06804	12664			47	1
200 kda cold-induced protein {12 kda fragment} [Triticum aestivum=wheat, Peptide Partial, 25 aa]/fragment of WCS200	AAB31284	2729	ND	ND	44	1
Plastocyanin, chloroplast precursor	P08248	15699	ND	ND	43	1
ribulose-1,5-bisphosphate-carboxylase [Euphorbia characias]	CAA49390	6304	ND	ND	40	1
ribulose-1,5-bisphosphate carboxylase oxygenase large subunit [Heteranthera limosa]	AAD09845	49484	ND	ND	40	2
ribulose 5-bisphosphate carboxylase, large subunit [Gaillonia yemenensis]	CAC19566	51401	ND	ND	40	2

Figures

Figure 1. Protein gel blot analysis using the WCS120 antibody on proteins soluble upon boiling from cold acclimated (CA) and non-acclimated (NA) cultivars Mironovskaya 808 (MIR) and Bezostaya 1 (BEZ).

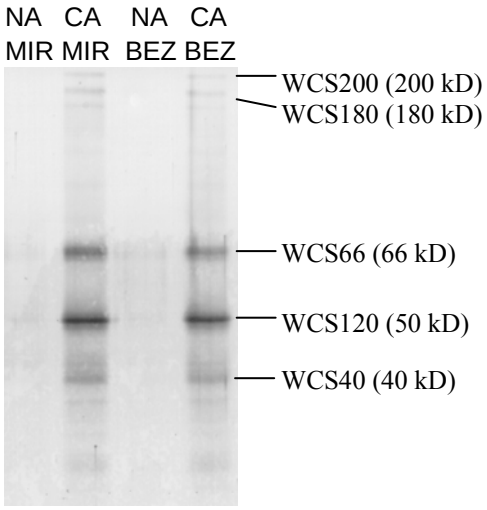


Figure 2. Comparison of 2-DE gels of proteins (soluble upon boiling) extracted from leaf tissue of non-acclimated (NA) and cold-acclimated (CA) winter wheat Bezostaya 1 (BEZ) and Mironovskaya 808 (MIR). 2-DE gels were silver-stained.

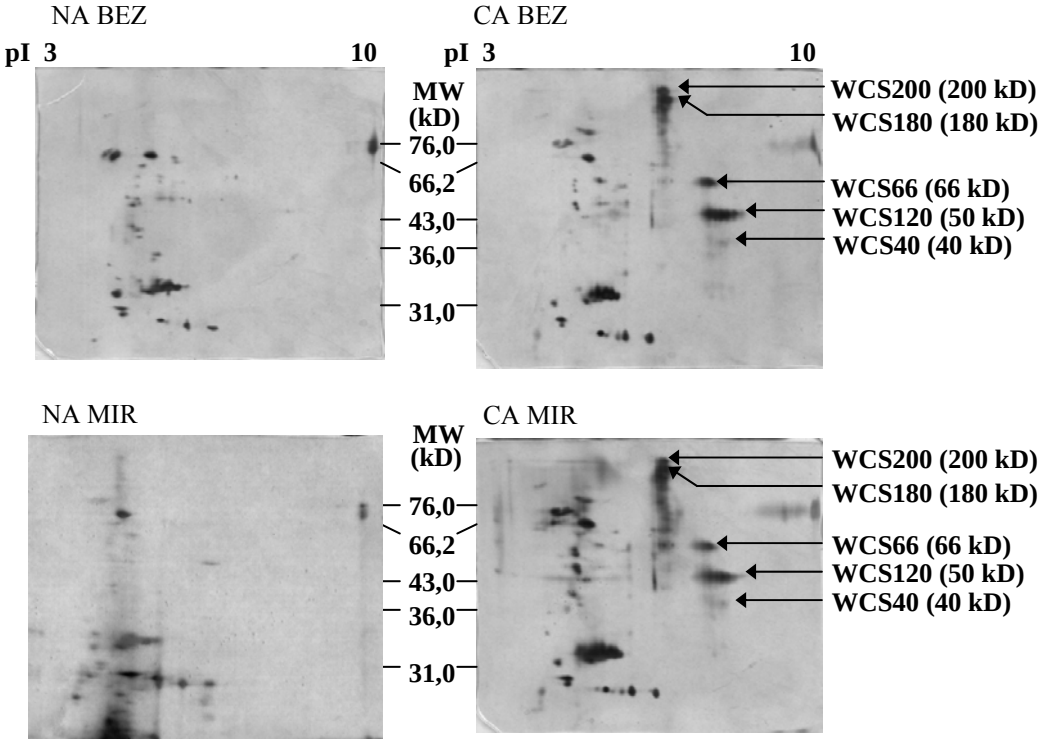
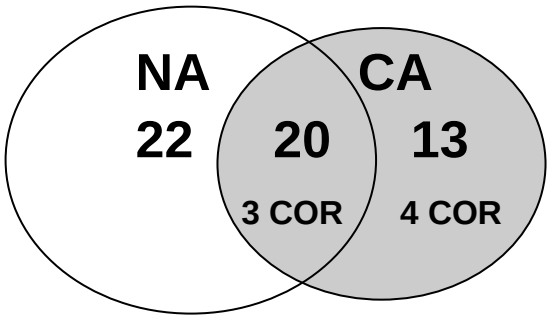


Figure 3. Results of protein identification by LC-MS/MS on total protein sample of non-acclimated (NA) and cold-acclimated (CA) leaves of cultivar Mironovskaya 808. The numbers represent total identified proteins including COR proteins which are separately presented by numbers of identified COR proteins.



Paper 4

WCS120 protein family and frost tolerance during cold-acclimation, de-acclimation and re-acclimation of winter wheat

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Abstract

We studied how long-term cold acclimation (up to 112 days), and how short (5 days) or long (14 days) de-acclimation, followed by re-acclimation of the winter wheat Mironovskaya 808, affected the level of WCS120 proteins, dry-weight content (DWC), and frost tolerance (FT) in leaves. Five members of WCS120 protein family were quantified by image analysis in protein gel blots or 2-DE (two-dimensional gel electrophoresis) gels. A typical response curve of FT during plant cold treatment, beginning with a steep increase, followed by maintenance at a high level of FT for several weeks, and then a gradual decrease, was observed, not only for the FT of leaves, but also for DWC and accumulation of WCS120 proteins in leaves. Using a mathematical model (the peak four parameter Weibull equation), it was demonstrated that the maximum DWC and accumulation of WCS120 proteins were reached at about the same time as the vernalization saturation of Mironovskaya 808, while the maximum of the leaf FT was about 14 days earlier. The DWC, content of WCS120 proteins, as well as the level of leaf FT decreased after plant de-acclimation. The longer the de-acclimation, the greater was the decrease of these traits. After 21 days of plant cold-re-acclimation, the re-accumulation of DWC and FT reached a similar level as in plants with the same time of cold treatment but without de-acclimation; while the WCS120 proteins accumulated at a lower level. The lower ability of WCS120 proteins to re-accumulate was detected in partially vernalized plants, while the ability to re-establish a high level of FT decreased in plants with fully saturated vernalization. From our results, we have formulated a hypothesis that developmental genes influence the ability to re-accumulate WCS120 proteins by partial vernalization of plants, while the ability to induce high FT is only influenced by the saturation of vernalization.

Keywords

cold acclimation, frost tolerance, re-acclimation, vernalization, WCS120 proteins, *Triticum aestivum*

Abbreviations

CA: cold acclimated, COR: cold-regulated, DA: de-acclimated, DWC: dry-weight content, FT: frost tolerance, LEA: late embryonic abundant, LT_{50}: lethal temperature, NA: non-acclimated, RA: re-acclimated, 2-DE: two-dimensional gel electrophoresis

Introduction

Both vernalization and frost tolerance (FT) in winter wheat are controlled by cold. Vernalization requires long-term exposure to cold temperature, while FT is induced immediately after plant exposure to cold treatment, and is lost very quickly after transferring plants back to normal conditions (Fowler et al. 1996a, Mahfoozi et al. 2001a, b). Vernalization prevents overwintering wheat from entering the frost-sensitive reproductive phase. Winter wheat in the vegetative phase is able to maintain a high level of FT in winter. Spring wheat without vernalization requirement enters the reproductive phase very quickly and is able to develop only a limited level of FT (Prášil et al. 2004).

Accumulation of some *Cor* (cold-regulated) transcripts and COR proteins during cold acclimation was found to be positively correlated with the level of FT in wheat and other cereals (e.g., Houde et al. 1992b, NDong et al. 2002, Kobayashi et al. 2004, Herman et al., 2006). The *wcs120* (wheat cold specific) gene family belongs to the Cor/Lea superfamily (Thomashow 1999), and encodes a group of highly abundant proteins ranging in size from 12 to 200 kDa (Houde et al. 1992a, Sarhan et al. 1997). This protein family is coordinately regulated by low temperature, and accumulates to high levels in freezing-tolerant wheat plants, as well as in other frost-tolerant species of the *Poaceae* (Fowler et al. 1996a, Sarhan et al. 1997, Vítámvás et al., 2007). WCS120 proteins share homology with the Lea D11 dehydrins, and are rich in both glycine and threonine, highly hydrophilic, soluble upon boiling, and have a pI above 6.5 (Fowler et al. 1996a, Thomashow 1999). During de-acclimation of cold-acclimated wheat, a rapid decrease of the *wcs120* gene products, as well as WCS120 proteins, was observed (Kobayashi et al., 2004).

The dynamics of frost tolerance in wheat grown in a controlled environment under uniform conditions has been described in a number of studies (Fowler et al.

1996a, Prášil et al. 2004, Limin and Fowler 2006). Plants exposed to low temperature start rapidly to increase their FT; then they gradually slow the rate of the change, until the maximum of FT is reached. After that, there is a gradual decrease in FT. Fowler et al. (1996a) and Mahfoozi et al. (2001a) observed that wheat plants reached the highest FT about the same time as vernalization saturation, measured by final leaf number, occurred. Moreover, the WCS120 proteins' accumulation in the plants of winter wheat also showed a peak at the similar time of cold acclimation (Fowler et al., 1996a). This supported the hypothesis that genes for vernalization requirement, which control the transition from the vegetative to the reproductive phase, also act in the control of genes affecting the expression of low temperature-induced genes, associated with the acquisition of FT (Fowler et al. 2001, Danyluk et al. 2003).

The re-acclimation studies (Fowler et al. 1996b, Mahfoozi et al. 2001a, Prášil et al. 2004) (i.e., when plants after acclimation at low temperature, and de-acclimation at high-temperature are again exposed to cold), showed that winter wheat plants are able to re-establish a high level of FT during re-acclimation; but only before their vernalization requirement has been satisfied. After the saturation of their vernalization requirement, their ability to re-acclimate (i.e., gain a high FT), was gradually lost. This methodological approach was used in this paper in order to study the relationship between the levels of WCS120 proteins, dry-weight content, and FT. The aim was to find if accumulation of the WCS120 protein family is related to the level of FT during plant acclimation, de- and re-acclimation, and if the levels of these proteins are altered in winter wheat, having been partially vernalized. As most of the published papers concentrated on determinations of WCS120 proteins in leaves (shoots), while the level of FT was evaluated at the level of whole plants (crowns), we also focused on the determination of FT in leaves (i.e., in the same plant organs (tissues) as the WCS120 proteins were evaluated).

Materials and methods

Plants

Seeds of winter wheat (*Triticum aestivum* L.) cultivar Mironovskaya 808 were kindly received from the Selgen a.s., Prague breeding company.

Growth conditions

The seeds were germinated at 20°C for 4 d. Then the seedlings were grown in soil at a constant temperature of 17ºC, under a 12 h photoperiod and at an irradiance of 400 μmol m^{-2} s^{-1}, provided by a combination of vapor lamps and high intensity discharge lamps (LU/400/T/40, Tungsram, Hungary) in a growth cabinet (Tyler, Hungary). Under these conditions, plants were grown up to either the first-leaf or third-leaf stage, depending on the type of experiment (vernalization or cold-acclimation). The same conditions were used for de-acclimation of plants following cold acclimation. Two experimental procedures were used.

Experiment I: Plants at the third-leaf stage were cold-acclimated (CA) at 2±1°C and a 12 h photoperiod for 112 d. They were sampled at varying times of acclimation (0, 7, 14, 21, 42, 63, 91 and 112 d). The 21-, 42- and 63-d-cold-acclimated plants were de-acclimated (DA) for 5 d (17°C, 12 h photoperiod), and thereafter re-acclimated (RA) for additional 21 or 28 d (2±1°C, 12 h photoperiod)

Experiment II: Plants at the first-leaf stage, achieved by progressive sowing in 21-d intervals, were acclimated at 2±1 °C and an 8 h photoperiod to obtain plants with 0, 21, 42 and 63 d of vernalization at one time. The plants were then transferred to 17°C and a 12 h photoperiod. Once they reached the third-leaf stage (about 14 d), they were again acclimated at 2±1°C and 12 h photoperiod for following 21 d.

In both experiments, the last two fully developed leaves of plants were sampled for the following analysis:

Dry weight content

The dry weight content (DWC = 100*DW/FW) was calculated using the ratio of dry weight (DW) to the fresh weight (FW) of plants. The DW was determined by lyophilization

of the leaves (-45°C, overnight) in a CRYODOS-50 Laboratory Freeze Dryer (Telstar Industrial, S.L.).

Frost tolerance

The level of FT in leaves was evaluated by a direct frost test (Prášil and Zámečník 1998). A set of six to eight leaf segments, of 1 cm length per tube, was exposed in two repetitions, to five different frost temperatures in five freezers for 8 hours. At the beginning, small ice crystals were added to the tubes with segments at -2ºC to ensure

extracellular freezing of the segments for two hours. Then the rate of cooling and thawing was 2°C h^{-1}. After thawing, 14 ml of de-ionized water was added to each tube, and the degree of frost injury was evaluated by the conductivity method (Prášil and Zámečník 1998). The lethal temperature LT_{50} (i.e., the temperature at which 50% of the leaves were killed) was calculated according to the model of Janáček and Prášil (1991).

Vernalization requirement

Days to heading (indicating the time of vernalization saturation) were determined for ten plants of each sampling day when half of the ear on the first tiller of a plant was emerging. The plants were grown in a glasshouse at 20±2°C and 16 h photoperiod (Prášil et al. 2004). The vernalization requirement was established as the number of days sufficient for full vernalization (i.e., the heading time was not significantly decreased by further vernalization).

Protein extraction

Proteins were extracted by TRIS buffer (0.1 M TRIS-HCl, pH 8.0 containing Complete™, EDTA-free protease inhibitor cocktail (Roche)) from frozen plant leaves, as described by Vítámvás et al. (2007). COR/LEA proteins are stable upon boiling and, therefore, the boiling step (15 min) was used to enrich these proteins in the sample. The protein concentration was measured according to Bradford (1976).

Protein analysis

Proteins were separated by SDS-PAGE on 10% gels (Laemmli 1970) or by 2-DE (O'Farrell 1975). Isoelectric focusing was run on pre-cast ReadyStrip IPG strips (pH 3-10) in PROTEAN IEF cell (Bio-Rad according to Instruction Manual (Bio-Rad). The proteins, soluble after boiling, were loaded into each of the IPG strips equally to 40 mg of FW leaves. The rehydration buffer contained 9.8 M urea and 4% CHAPS. The condition of isoelectric focusing was 35,000 V-hr. The focused proteins were then separated, in the second dimension, on 10% SDS-PAGE.

The separated proteins were identified by a protein gel blot analysis using the anti-WCS120 polyclonal antibody (Houde et al., 1992a) or anti-dehydrin polyclonal antibody (against the consensus K-segment, Close et al. 1993). The proteins were loaded into each well equally to 0.4 mg of FW leaves. The proteins were electrophoretically transferred to nitrocellulose (0.45 μm, Pharmacia Biotech). After

blocking with nonfat dry milk (3 %) in TBS, the membrane was incubated with a 1:20,000 dilution of WCS120 antibody or with 1:1000 dilution of anti-dehydrin antibody. After washing with TBS containing 0.05 % Tween-20 and 0.2 % Triton X-100 (Sigma), the secondary antibody (GAR-AP conjugate, Bio-Rad) was applied at 1:3,000 dilution. The complex of proteins and antibodies was visualized by BCIP/NBT staining (Bio-Rad). SDS-PAGE Standards, broad range, (Bio-Rad) were used for an estimation of molecular weight (MW). The software ElfoMan 2.6 (Semecký, Czech Rep.) was used for the image evaluation of one-dimensional gels or W-blots. All images were captured by ColorPage-SP2 (Genius). 2D SDS-PAGE Standards (Bio-Rad) were used for the calibration of MW and pI.

Image analysis of 2-DE gels

The image analysis was used to quantify the density of the protein spots on the 2-DE gels according to Vítámvás et al. (2007). The images were evaluated by the image analysis system LUCIA G v.4.80 (Laboratory Imaging Prague, Czech Republic) equipped with a Dual Pentium MMX and Matrox Magic frame grabber.

The statistical evaluation was carried out based upon a multiple range test of averages (LSD at the 5% level), calculated from at least 3 repetitions (Unistat version 5.1, Unistat Ltd., London, UK).

The peak four parameter Weibull equation was used to describe the relationship between LT_{50}, dry weight content, amount of WCS120 proteins (y) and days of acclimation (x) according to Fowler and Limin (2004):

$$y = a\left(\frac{c-1}{c}\right)^{\frac{1-c}{c}}\left[\frac{x-x_0}{b}+\left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]^{c-1} e^{-\left[\frac{x-x_0}{b}+\left(\frac{c-1}{c}\right)^{\frac{1}{c}}\right]^{c}} + \frac{c-1}{c}$$

, where regression coefficient *a* is the estimate of peak value (y) and coefficient x_0 estimate of the days to reach it. Non-linear regression procedures outlined by SigmaPlot (8.0) were used to provide least squares estimates of the regression coefficients in this equation.

Results

WCS120 proteins, LT50 and vernalization of wheat during acclimation and re-acclimation (Experiment I)

Plants were CA for 112 d. At 21, 42 and 63 d of acclimation the parts of plants were returned to 17°C for 5 d (de-acclimation) and then were exposed again to cold (re-acclimation). The accumulation of WCS120 proteins during cold-acclimation, de-acclimation (excluding sample DA after 21 d of acclimation) and re-acclimation was achieved by protein gel blot analysis of proteins soluble upon boiling (Figs. 1 and 2). Both types of antibodies (anti-WCS120 or anti-dehydrin; in figure 1B it is shown for anti-dehydrin) were bound to the same five bands that were very visible after cold-acclimation and re-acclimation on protein gel blots. These five protein bands were identified as WCS120 proteins according to their molecular weights – WCS200 (MW 200 kDa), WCS180 (MW 180 kDa), WCS66 (MW 66 kDa), WCS120 (MW 50 kDa) and WCS40 (MW 40 kDa). It means that all five of the most abundant members of WCS120 proteins were detected in plants exposed to cold. Furthermore, the WCS120 proteins were also very noticeable on silver-stained 1-DE gels (Fig. 1A). In all samples from cold treatment, the WCS120 protein reached the highest accumulation level; WCS66 was second, and WCS40 had the lowest accumulation.

The non-acclimated (NA) samples showed only very low levels of WCS120 protein. In the CA samples, all members of WCS120 proteins showed very similar trends in their accumulation (Fig. 2). Then, their levels decreased up to 112 d of acclimation. After 5 d of plant de-acclimation the amount of WCS120 proteins rapidly decreased in all treatments. The samples from DA leaves had all WCS120 proteins, but only present in very low amounts (Fig 2). After re-acclimation the amount of WCS120 proteins in the leaves increased again; however, they did not reach the same levels as plants exposed to the same length of cold period, but without de-acclimation. The level of WCS120 proteins in the RA sample, after 5 d of de-acclimation, was similar in partially vernalized plants, contrary to the significantly lower levels in plants after fully saturated vernalization.

Days to heading, lethal temperature (LT_{50}), dry-weight content (DWC) as well as the sum of WCS120 proteins during cold-acclimation, de-acclimation and re-acclimation are shown in Fig. 3. The parameters of the curves for CA plants are given in Tab. 1. The coefficients of determination (R^2) of the LT_{50} (0.86), WCS120 (0.99) and

DWC (0.97) curves showed that the calculated values were close to the measured values. The heading time decreased progressively, up to 63 d of acclimation; beyond that, it did not significantly change, indicating that the vernalization requirement of the cultivar had been saturated in 63 d of acclimation. At about this time in the acclimation, the content of WCS120 proteins showed a maximum accumulation (the peak value was 55 d), as well as the accumulation of DWC (61 d) (Fig. 3, Tab. 1). On the other hand, the peak of frost tolerance (42 d for minimum LT50) was obtained about 14 d before the maximum accumulation of WCS120 proteins and saturation of the plant vernalization requirement (Tab. 1).

After 5 d of plant de-acclimation, DWC and FT rapidly decreased in all treatments. The levels of DWC and FT were significantly higher in the DA leaves contrary to the NA leaves, similar to the amounts of WCS120 proteins. After the re-acclimation, the DWC and LT_{50} reached a similar value as those plants with the same cold treatment but without de-acclimation. On the other hand, the re-established values of WCS120 proteins were significantly lower in the RA samples than their values in the CA ones (Fig. 3).

WCS120 proteins and LT50 in wheat vernalized for different time (experiment II)

The previous experiment (Experiment I) demonstrated that the dynamics of FT (LT50) as well as the content of WCS120 proteins differed during cold-acclimation and re-acclimation. Therefore, in the following experiment we studied how these two traits are influenced in plants that had been vernalized from 0 to 63 d before re-acclimation.

WCS120 proteins were identified on 2-DE gels (Fig. 4) according to their pI, MW values, with detection by anti-WCS120 antibody on protein gel blots (not shown); and then were quantified by image analysis (Tab. 2). Before re-acclimation, only a small amount of WCS120 protein was detectable in the leaves of partially vernalized plants (Tab. 2). After re-acclimation, all members of WCS120 proteins were detected; the highest amounts were found in plants without previous vernalization. The WCS120 protein reached the highest level, when compared to the other members of WCS120 proteins. WCS66 protein was second and WCS180 in third position, considering the degree of their accumulation. However, their accumulation progressively decreased from 0- to 63-d-vernalized plants. The level of both WCS200 and WCS40 was very low in all samples, so their differences were usually insignificant; however, it was possible

to see that their level was lower in 63-d-vernalized plants, in comparison with non-vernalized plants (Tab. 2).

Before re-acclimation (i.e., after the plant growth at high temperature - about 14 d), the LT_{50}s of leaves in all pre-vernalized plants were insignificant, and very highly indicating that they were completely de-acclimated. However, they were able to induce a high level of FT after re-acclimation (Fig. 5), excluding the plants with a saturated vernalization requirement, which showed a significantly high LT_{50} (-16.1 ºC), i.e., lower FT. The heading day progressively decreased with the partial vernalization of plants (Fig. 5), as well as their accumulation of WCS120 proteins after re-acclimation, indicating that they gradually reduced their ability to re-accumulate the WCS120 proteins in leaves with gradual vernalization of plants.

Discussion

In this study, we have dealt with the impacts of a long-term cold acclimation, and both short (5 d) or long (14 d) de-acclimation, followed by the re-acclimation of winter wheat, on the levels within leaves of WCS120 proteins, DWC and FT (LT50).

Houde et al. (1992b) proposed, for the first time, that the accumulation of proteins of the WCS120 family act as an important marker of the actual level of plant FT. Since then, many other studies have used the WCS120 protein as a marker of FT for distinguishing wheat cultivars with different levels of FT, i.e., winter and spring cultivars (Fowler et al. 1996a; NDong et al. 2002; Kobayashi et al. 2004). In our previous study (Vítámvás et al. 2007), we even used these proteins for the detection of fine distinctions between two highly frost-tolerant winter wheat cultivars (Mironovskaya 808 and Bezostaya 1), after 21 d of cold treatment.

Here, we were able to distinguish and quantify all five members of WCS120 protein family by two different methods – protein gel blot analysis (Fig. 1) and 2DE analysis (Fig. 4). The most abundant protein was WCS120 protein, followed by WCS66 and WCS180. The lowest levels were shown by WCS200 and WCS40 proteins, corresponding to published data (Vítámvás et al. 2007). All rapidly accumulated after cold-acclimation or re-acclimation and rapidly decreased after de-acclimation; however, the sum of all WCS120 proteins better expressed the differences between the treatments.

We obtained a typical response curve of FT during plant cold treatment; beginning with a steep increase in the first 21 d, followed by the maintenance of a high level of FT in the interval between 21 to 70 d, and then a gradual decline of FT. This described the dynamics of the FT of leaves (only known before this for the FT of wheat plants (Fowler et al. 1996a, Fowler and Limin 2004, Prášil et al. 2004)); and was also shown for DWC and the accumulation of WCS120 proteins in leaves (Fig. 3). These three mentioned traits, determined in the leaves, were compared with vernalization of the plant, preceded by cold treatment. The fully saturated vernalization requirement, founded here for 63 d acclimated plants of cultivar Mironovskaya 808, was similar with the previous published findings (Prášil et al. 2004, 2005).

Using the same mathematical model as published by Fowler and Limin (2004) for long-term cold treatment, showed support that the maximum of the leaf FT was about 14 d earlier than the vernalization requirement of the plant (Fig. 3). The effect of photoperiod sensitivity upon accumulation of FT, even before vernalization saturation, was described in winter wheat cold-acclimated under a long day (Mahfoozi et al. 2001b). This also could explain the earlier accumulation of FT in our experiments in Mironovskaya 808, a cultivar highly sensitive to the photoperiod (Košner and Pánková, 1998). On the other hand, the discrepancy between the maximum of FT (calculated as peak of the Weibull equation) and the saturation of vernalization did not mean that FT abruptly decreased before saturation. A significant decrease of the leaf FT, found here, as well as of the plant FT (published by Prášil et al. (2004)), were observed 14 to 21 d after the saturation of the Mironovskaya vernalization requirement.

The curves (dynamics) of DWC and amounts of WCS120 proteins for long-term cold-acclimation were very similar with the curve of level of FT (LT50) (Fig. 3). Furthermore, after a short 5 d de-acclimation, the DWC and content of WCS120 proteins decreased, as well as the level of FT. The same dynamics of the content of WCS120 proteins and of LT_{50} were observed for wheats and ryes by Fowler et al. (1996a). A rapid decline in the content of WCS120 protein during 5 d of de-acclimation was detected in Mironovskaya 808 by Kobayashi et al. (2004). Comparing the results between both experiments showed that the longer the de-acclimation (about 14 d in Exp. II, compared to 5 d in Exp. I), the greater the decrease of WCS120 proteins, followed by lower re-accumulation of WCS120 proteins. For instance, after 5-d-de-acclimation of 42-d-acclimated plants some level of WCS120 proteins was detected

(Fig. 3); while after about 14 d of de-acclimation of 42-d-pre-acclimated (pre-vernalized) plants the WCS120 proteins nearly completely disappeared (Tab. 2).

Relationships between LT50 and DWC (usually mentioned as water content WC = 100 – DWC) have been published many times (e.g. review Sakai and Larcher, 1987), and that the changes in DWC could be explained by changes in water and/or dry weight (Prášil et al. 2001). Here, we assume that changes (dry weight accumulation), mainly in the sugar metabolites, are due to photosynthesis under the light conditions in mature leaves.

Moreover, in our Experiment I, the differences between the three traits studied appeared after re-acclimation of plants, when the DWC and LT50 reached similar values as in those plants with the same cold treatment time, but without de-acclimation; while the WCS120 proteins did not (Fig. 3). This indicated that changes in LT50 were more closely related to changes in DWC, than in the content of WCS120 proteins. It is interesting that it is possible to detect the lower ability of WCS120 proteins to re-accumulate after the saturation of vernalization of wheat plants, as well as before saturation. This was confirmed in Experiment II with the re-acclimation of plants at different times of pre-vernalization (where the ability to induce a high level of FT only decreased in plants after saturation of vernalization); while the ability to accumulate a high level of WCS120 proteins decreased by partial vernalization of plants, measured by days to heading. A lowered ability to re-establish a high level of FT after the saturation of vernalization in winter wheats was shown by Fowler et al. (1996b), Mahfoozi et al. (2001a), Prášil et al. (2004). From our results, we formulate the hypothesis that developmental genes (e.g. vernalization genes) influence the ability to re-accumulate WCS120 proteins by partial vernalization of plants, while the ability of wheat to induce high FT is influenced only by saturation of vernalization.

Acknowledgments

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Tables

Table 1. Extreme (peak) value of lethal temperature (lowest LT_{50}), dry weight content (highest DWC), content of WCS120 proteins (highest WCS120) (= estimate of parameter **a** of peak four-parameter Weibull equation) in leaves and days of plant Acclimation (at 2ºC) to reach the extreme value (= estimate of parameter $\mathbf{x_0}$ of peak four-parameter Weibull equation). SE = standard error.

	Extreme value	SE	Acclimation (d) *
LT50	-23.5 °C	1.2	41.5a
DWC	30.9 %	0.6	60.6b
WCS120	93.7 %	1.8	44.4b

* Values denoted with the same letter are not significantly different.

Table 2. Integral density of WCS120 proteins (in %) gained by image analysis of 2-DE gels in leaves before and after re-acclimation (21 d at 2ºC) of plants with different time of previous vernalization (0, 21, 42 and 63 d at 2ºC), achieved by progressive sowing. The sum of the integral density of all WCS120 spots in the re-acclimated plants without previous vernalization (0 d) was set to 100 %. The average density for each protein was calculated from 3 - 4 independent repetitions (2-DE gels). ND = non-detected.

	Integral Density (%)*							
	Before re-acclimation				After re-acclimation			
Vernalization (d)	0	21	42	63	0	21	42	63
protein								
WCS200	ND	ND	0.09	ND	6.2a	6.5a	5.9a	2.7b
WSC180	ND	ND	0.08	ND	17.6a	14.5a	8.0b	1.9c
WCS66	ND	ND	ND	ND	20.3a	14.1b	2.7c	2.7c
WCS120	0.19a	0.2a	0.15a	ND	51.1a	46.1a	27.8b	17.0c
WCS40	ND	ND	ND	ND	4.8a	1.0a	2.3a	0.7a
Sum	0.19a	0.2a	0.32a	ND	100.0a	82.4b	46.8c	25.0d

* Values in the same line denoted with the same letter are not significantly different within the same treatment (i.e. before or after re-acclimation).

Figures

Figure 1. Silver stained gel (a) and W-blot with anti-dehydrin antibody (b) of leaf heat-stable proteins sampled during cold acclimation (0-112 d at 2ºC), de-acclimation (5 d at 17ºC) and re-acclimation (21 or 28 d at 2ºC) of Mironovskaya 808 plants; detected WCS120 proteins (WCS40 to WCS200); K, Kaleidoscope Prestained Standards, broad range (Bio-Rad); St, SDS-PAGE standards, broad range (Bio-Rad); 0-112 indicates number of days of cold-acclimation; D42 and D63 de-acclimation after 42 and 63 d of cold acclimation; R21, R42 and R63 re-acclimation after 21, 42 and 63 d of cold-acclimation and 5 d of de-acclimation.

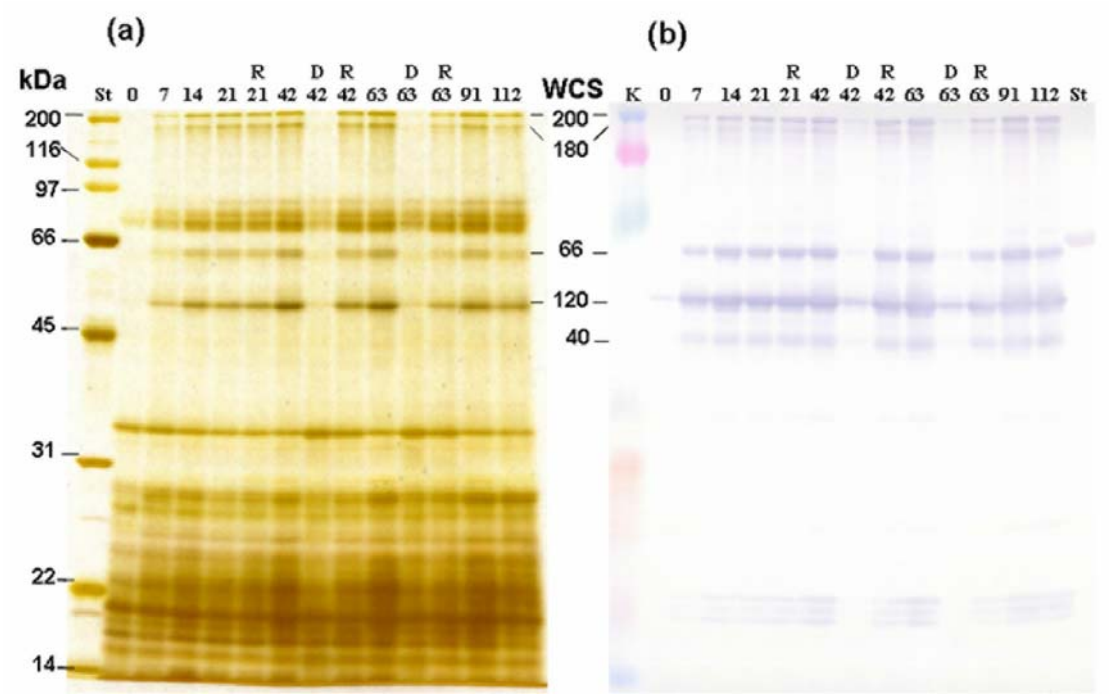


Figure 2. Accumulation of WCS120 proteins (from WCS40 to WCS200 protein) in leaves, during: cold acclimation (0-112 d), de-acclimation (D42; D63) and re-acclimation (R21; R42; R63) of plants. The descriptions are the same as with Fig. 1. The highest sum of integral density of all WCS120 bends of a sample was setting to 100 % and the average density for each protein was calculated from 6 independent repetitions (W- blots). Values of the same protein denoted with the same letter are not significantly different (a-f, letter for the each member of WCS120 protein; A-E, for the sum of all proteins).

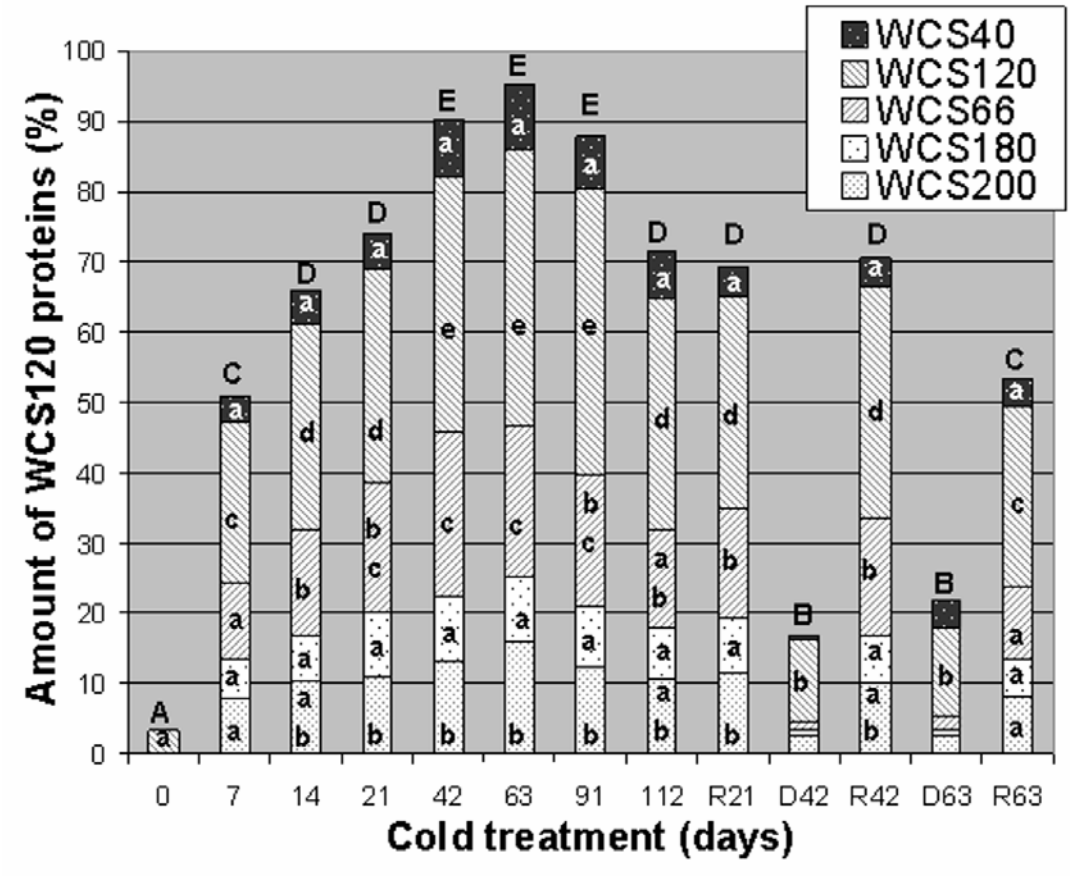


Figure 3. Dynamics of LT_{50}, dry weight content (DWC) and accumulation of the WCS120 proteins (WCS120) in leaves, and days to heading (heading) in plants during: cold acclimation (0-112 d at 2ºC), de-acclimation (5 d at 17ºC) and re-acclimation (21 or 28 d at 2ºC). The sum of all WCS120 was calculated as in Fig. 2.

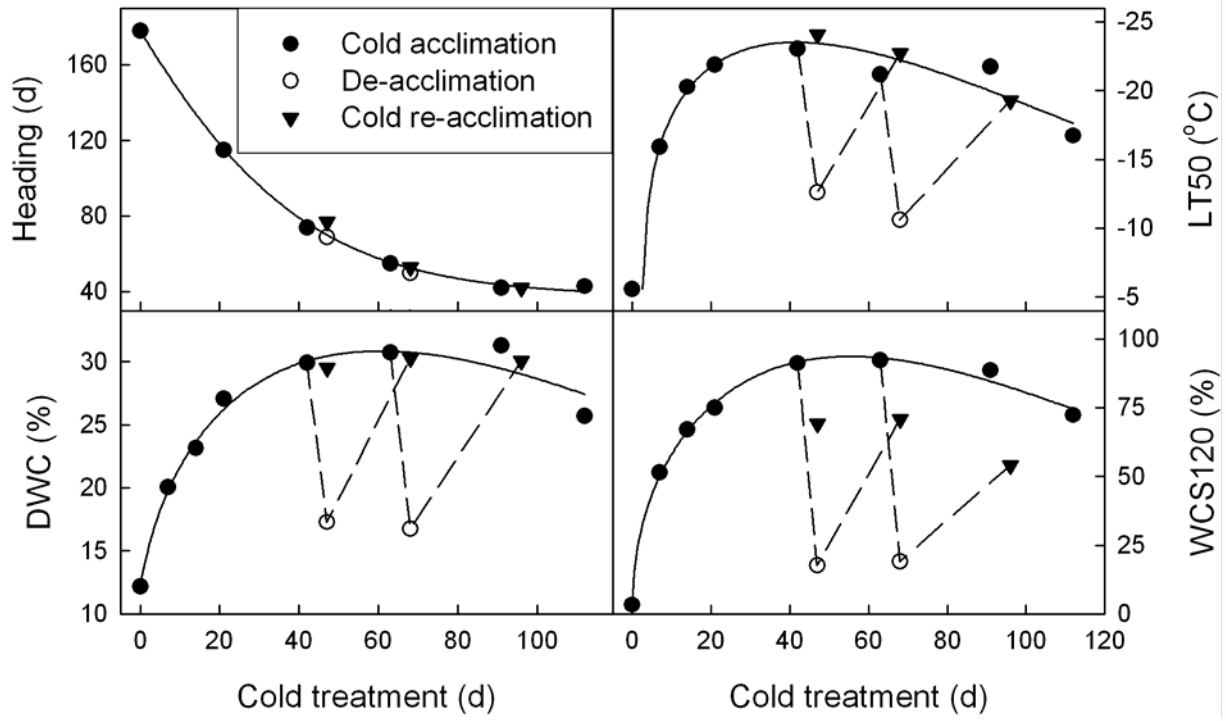


Figure 4. Comparison of leaf proteins, soluble upon boiling, after the re-acclimation (21 days at 2 ºC) of plants with different times of previous vernalization (0, 21, 42 and 63 d at 2ºC), achieved by progressive sowing. 2-DE gels were silver stained and marked according to days of previous vernalization. The WCS120 proteins were highlighted in the 2-DE gel "0" (1 = WCS200, 2 = WCS180, 3 = WCS66, 4 = WCS120, 5 = WCS40). The 2-D gels "63" also showed the 2D SDS-PAGE marker (Bio-Rad) (indicated by arrows).

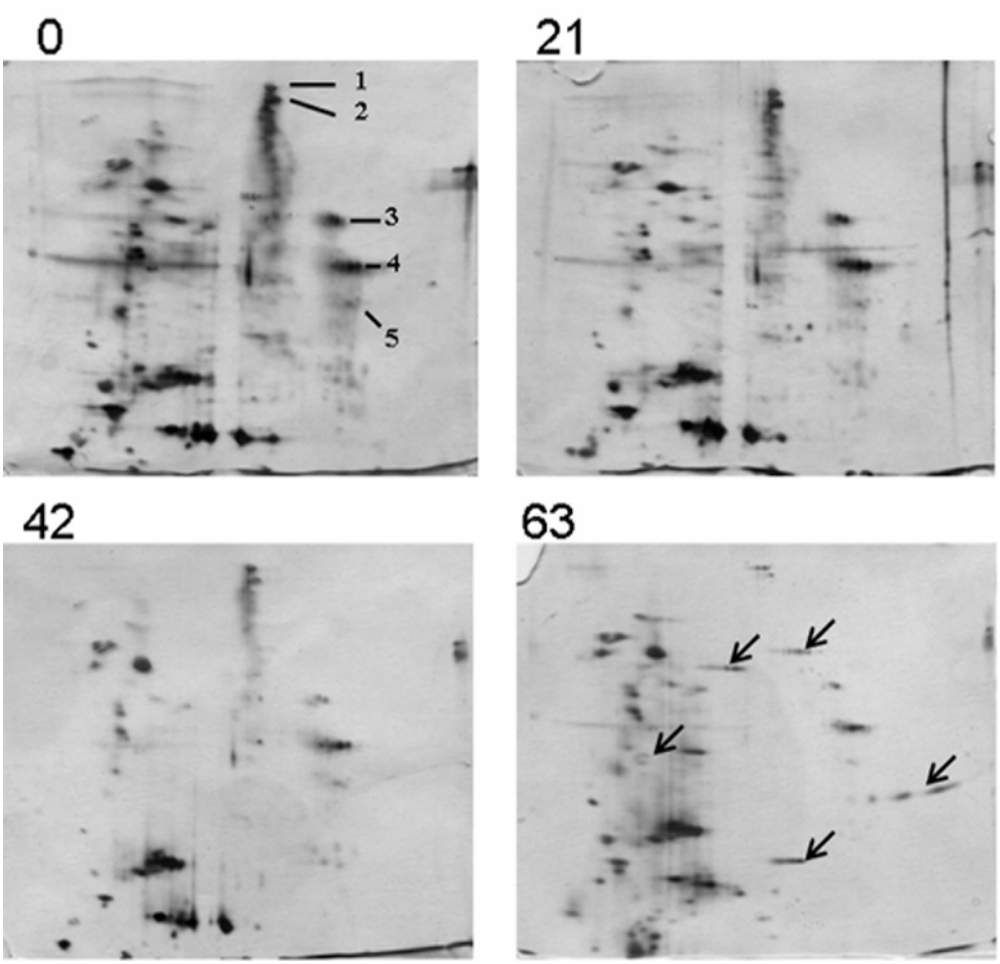
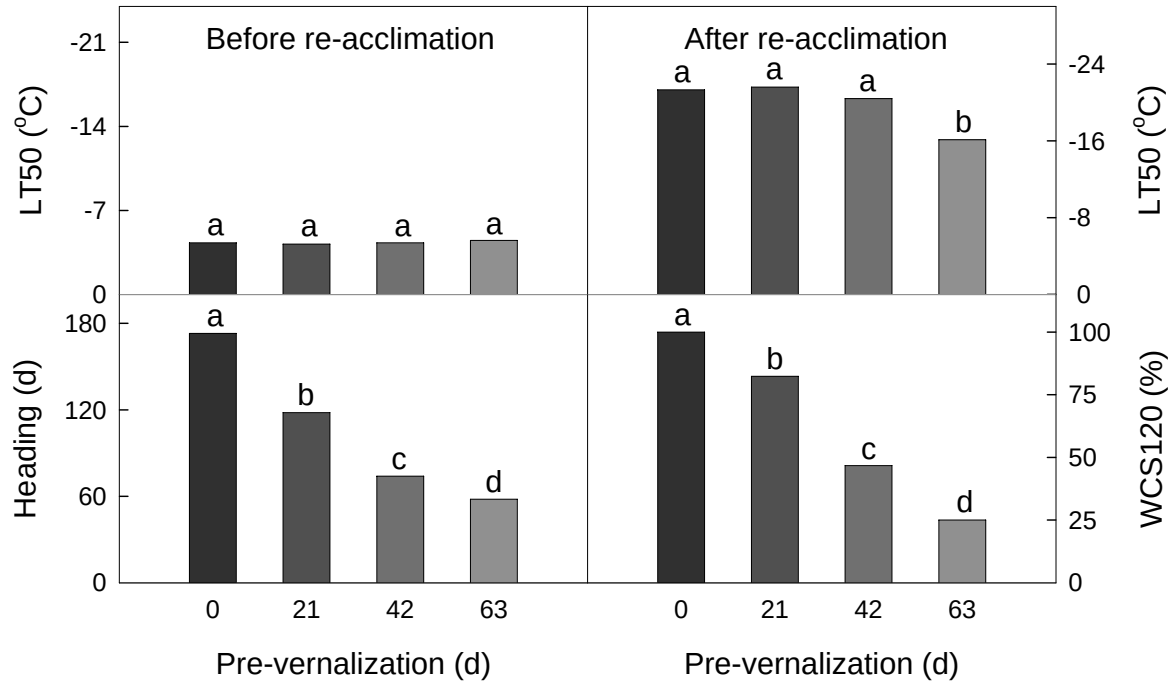


Figure 5. The comparison of days to heading in plants and leaf LT_{50} before, LT_{50} after, and content of WCS120 proteins after re-acclimation of plants with different times of previous vernalization (0, 21, 42 and 63 d at 2ºC), achieved by progressive sowing. The sum of all WCS120 was calculated as in Tab. 2. Values in the same diagram denoted with the same letter are not significantly different.



Paper 5

Temperature-dependent accumulation of dehydrins in wheat and barley cultivars with different frost tolerance

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Abstract

Using five cultivars of wheat and two of barley, with different abilities to tolerate frost, we have shown that dry weight content, frost tolerance, induction of dehydrins, as well as their accumulation in the leaves were tolerance- and temperature-dependent. The plants were grown at 25, 17, 9 or 4°C, and both the cold-regulated dehydrins in wheat (WCS120 proteins) and in barley (DHN5 = WCS120 ortholog), were detected by the same anti-dehydrin antibody. The lower the growth temperature, the higher was the level of frost tolerance, dry weight content and dehydrins' accumulation, in all cultivars. However, winter and spring wheats were differentiated according to the threshold temperatures for the induction of expression (i.e., response: present / absent), as well as for the accumulation (i.e., response: greater / less) of the dehydrins. The barleys only were differentiated according to the threshold temperature for the accumulation of the dehydrin, and at a lower temperature (9°C) than the wheats (17°C). Moreover, the highly frost-tolerant (as opposed to the lower-tolerant or sensitive) wheat cultivars already accumulated a higher level of WCS120 proteins at 17°C (i.e., at the higher temperature when it was not possible to differentiate them by a frost test).

Keyword index: *Triticum aestivum*, *Hordeum vulgare*, threshold temperature, WCS120, DHN5, frost tolerance

Introduction

Frost tolerance (FT) of crops is induced, immediately after the plant's exposure to cold treatment. It has been found that a rapid temperature decline leads to the expression of *Cor* (cold-regulated) genes in cereals (reviewed in Thomashow 1999, and in Cattivelli *et al.* 2002).

Among the *Cor* genes, it has been shown that the *wcs120* (wheat cold specific) gene, which belongs to the group of genes that encode dehydrins, can be considered as a marker of freezing tolerance (Houde, Dhindsa & Sarhan 1992). The *wcs120* gene is a member of the *wcs120* gene family which encodes proteins (of apparent molecular weights of 200, 180, 66, 50 and 40 kDa) that are all cold-inducible and labelled as the WCS120 protein family; here called WSC120 proteins (for review, see Sarhan, Ouellet & Vazquez-Tello 1997). In barley, it has been found by many authors (e.g., Van Zee *et al.* 1995, Bravo *et al.* 1999, Zhu *et al.* 2000) that the *wcs120* ortholog, the *dhn5* (dehydrin 5) gene, is induced by cold. In the promoter of *wcs120* (Ouellet, Vazquez-Tello & Sarhan 1998) and *dhn5* (Choi, Zhu & Close 1999) genes, a C-repeat (CRT), the dehydration-responsive element (DRE), was found; and therefore, these genes are also members of the CBF (CRT/DRE binding factor) regulon and components of the CBF cold-response pathway (reviewed in Van Buskirk & Thomashow 2006).

In cold-acclimated cereals, higher transcription levels (Vágújfalvi *et al.* 2005) or longer expression durations (Skinner *et al.* 2005) of *Cbf* genes were observed in frost-tolerant (winter) cultivars, than in frost-sensitive (spring) cultivars. The higher levels of expression of *Cor* genes and/or accumulation of COR proteins in frost-tolerant, compared to in frost-sensitive cultivars, grown under cold conditions, have been described in several studies, e.g., Fowler *et al.* (1996), Danyluk *et al.* (1998), Danyluk *et al.* (2003) and Kobayashi *et al.* (2004). Furthermore, winter wheats with different levels of FT have been distinguished by the levels of WCOR410 proteins (Danyluk *et al.* 1998) as well as WCS120 proteins (Vítámvás *et al.* 2007). However, not all published results have indicated that differences in the amount of COR proteins were significant between different frost-tolerant barley cultivars, after cold-acclimation (Van Zee *et al.* 1995, Crosatti *et al.* 1995). Moreover, the amount of COR proteins in different winter barley cultivars, grown under low temperatures, did not correlate with the level of FT (Bravo *et al.* 1999).

Crosatti *et al.* (1995, 1996) studied changes in the accumulation of chloroplast-localized products of the c*or14* gene in wheat cultivars and its homolog in barley, cultivated under different temperatures, and found that the threshold induction temperature for COR14 proteins is lower in the frost sensitive cultivars than in the frost tolerant ones. Moreover, they observed that the temperature threshold at which the *cor14b* gene became expressed was higher in wheat than in barley (Galiba *et al.* 2001). However, very little is known yet about the temperature- and tolerance-dependent

dynamics of other *Cor* genes' expression. Up to now, there has not been any published evidence that the different frost-tolerant cultivars have different threshold temperatures for the induction of dehydrins, such as WCS120 proteins in wheat or DHN5 in barley (Kosová, Vítámvás & Prášil 2007).

In our previous work (Vítámvás *et al.* 2007), we distinguished two winter wheat cultivars grown at 2°C, there having used a densitometric analysis of WCS120 spots on 2DE-gels. However, we have found a slight accumulation of WCS120 at 17°C in both winter wheat cultivars; whereas, no accumulation of this protein at the same temperature in the spring wheat cultivar have been detected (Vítámvás *et al.* unpublished). These results have led us to the question of whether the expression of dehydrins, related to cold-acclimation, is induced at a higher temperature in the highly frost-tolerant cultivars; while at a lower temperature and with a lower rate in the low-tolerant cultivars. Thus, our aim was to study if the accumulation of WCS120 proteins in wheats and DHN5 in barleys could be related to the level of FT of the cultivars grown at different temperatures. To get more information on the development of plant responses to different temperature (cold) treatments, the dry weight content (DWC), generally indicating the accumulation of dry-weight in mature leaves (Prášil, Kadlecová-Faltusová & Faltus 2001), was also assessed.

Materials and Methods

Plant material

These experiments were conducted with the winter barley (*Hordeum vulgare* L.) cultivar Luxor, the spring barley cultivar Atlas 68 (Atlas), plus the four winter wheat (*Triticum aestivum* L.) cultivars Mironovskaya 808 (M808), Šárka, Zdar, and Bill, as well as the one spring wheat cultivar Sandra. The seeds of the cultivars were obtained from the breeding company Selgen a.s., Prague, Czech Republic.

Growth conditions

The seeds were germinated at 20°C for 3 d. Then, the seedlings were grown in a growth cabinet (Tyler, Hungary), with a 12 h photoperiod (irradiance of 400 μmol m^{-2} s^{-1} provided by a combination of vapour lamps and high intensity discharge lamps (LU/400/T/40, Tungsram, Hungary)) and at several temperatures (25, 17, 9 or 4°C). Two experimental procedures were used.

In the first experiment, the seedlings were grown and developed their leaves (up to the three-leaf stage) at one of the experimental temperatures (i.e., 10 days at 25°C, 3 weeks at 17°C, or 5 weeks at 9°C). Because of the very long time to reach the three-leaf stage at 4°C, at first, the plant grew 2 weeks at 17°C and then 4 weeks at 4°C (in order to develop the third leaf at 4°C).

In the second experiment, the seedlings developed up to the three-leaf stage at 17°C for 3 weeks, and then were exposed to 9°C for a further 2 weeks.

In both experiments, the last fully developed leaf of the plants was sampled for the analysis that following.

Dry weight content

The dry weight content (DWC = 100*DW/FW) in the leaves was calculated as the ratio of dry weight (DW) to fresh weight (FW). DW was determined by lyophilization (-45°C, overnight) of the leaves in a Laboratory Freeze Dryer CRYODOS-50 (Telstar Industrial, S.L.).

Frost tolerance

The level of FT in the cultivars was evaluated by a direct frost test (Prášil & Zámečník 1998). A sets of six to eight leaf segments, of 1 cm length per tube, were exposed in two repetitions to five different frost temperatures in five freezers for 8 hours. Thereafter, small ice crystals were added to the tubes with segments at -2ºC, to ensure extracellular freezing of the segments for two hours. The rates of cooling and thawing were at 2°C h^{-1}. After thawing, 14 ml of deionised water was added to each tube, and the degree of frost injury was evaluated by the conductivity method (Prášil & Zámečník 1998). The lethal temperature LT_{50} (i.e., the temperature at which 50% of the leaves were killed) was calculated according to the model of Janáček & Prášil (1991).

Protein extraction

Proteins were extracted by TRIS buffer (0.1 M TRIS-HCl, pH 9.0, containing Complete™, EDTA-free protease inhibitor cocktail (Roche)) from frozen plant leaves as described by Vítámvás *et al.* (2007). Dehydrins are stable upon boiling, and therefore, a boiling step (15 min) was used to enrich the proteins in the sample. The protein concentration was measured according to Bradford (1976).

Protein analysis

Proteins were separated by SDS-PAGE on 10% gels (Laemmli 1970). Samples were loaded into each well equally to 0.4 mg of FW leaves. Afterwards, the proteins were identified by a protein gel blot analysis using the polyclonal anti-dehydrin antibody (against the consensus K-segment (Close, Fenton & Moonan 1993)). The proteins were electrophoretically transferred to nitrocellulose (0.45 μm, Pharmacia Biotech). After blocking with nonfat dry milk (3%) in TBS, the membrane was incubated with a 1:1000 dilution of anti-dehydrin antibody. After washing with TBS, containing 0.05% Tween-20 and 0.2% Triton X-100 (Sigma), the secondary antibody (GAR-AP conjugate, Bio-Rad) was applied at a dilution of 1:3000. The complex of proteins and antibodies was visualized by BCIP/NBT staining (Bio-Rad). SDS-PAGE Standards, broad range (Bio-Rad), were used for the estimation of molecular weight (MW). ElfoMan 2.6 software (Semecký, Czech Rep.) was used for the evaluation of the images of the SDS-PAGE gels or W-blots. All images had been captured using ColorPage-SP2 (Genius).

The statistical evaluation was carried-out based on a multiple range test (LSD at the 5% level) of averages, calculated from at least 3 repetitions (Unistat version 5.1, Unistat Ltd., London, UK).

Results

WCS120 proteins, LT_{50} and DWC in wheat leaves, developed at different growth temperatures

The spring wheat cultivar (Sandra), as well as four winter wheat cultivars (Bill, Zdar, Šárka, and M808), with differing abilities to develop frost tolerance were used to determine the differences in accumulation of WCS120 proteins, DWC, as well as level of frost tolerances (LT_{50}), after cultivations under different temperatures (25, 17, 9 or 4°C).

The accumulations of dehydrins in the leaves were detected by protein gel blot analysis of the proteins soluble upon boiling (Fig. 1). The polyclonal anti-dehydrin antibody bound to the most abundant members of WCS120 proteins, which were identified according to their molecular weights: WCS200, WCS180, WCS66, WCS120 and WCS40 (Fig.1B). Furthermore, the WCS120 proteins were also noticeable on silver-stained 1-DE gels (Fig. 1A).

In all of the wheat samples with visible WCS120 proteins, WCS120 reached the highest amount, WCS66 was second (with the exception of Zdar), and WCS40 had the lowest amount. WCS66 was not present in any immunoblots of winter wheat Zdar, and therefore, Zdar had reduced values of the sum of all WCS120 proteins. However, the anti-dehydrin antibody recognized one band having a molecular weight of about 26 kDa, but only in the leaves of Zdar cultivated at 4°C (Fig. 1B). This protein accumulated to levels lower than WCS40.

A strong interaction between growth temperature, levels of FT, DWC and WCS120 proteins' accumulation was observed (Fig. 2). The differences in the accumulation of the sum of all WCS120 proteins, extracted from plants grown at different temperatures, showed almost the same trend as the WCS120 protein alone. In all cultivars cultivated at 25°C, the WCS120 proteins were not detected, and FT and DWC showed the lowest and identical values.

When the wheats were grown at 17°C, the highly frost-tolerant cultivars M808 and Šárka accumulated WCS120 proteins, contrary to the undetectable levels of WCS120 proteins in the low frost-tolerant Zdar and Bill, as well as frost-sensitive Sandra. Correspondingly, the value of LT_{50} was slightly lower in the M808 cultivar than in the others. However, the LT_{50} of the leaves of the other cultivars had a similar value, and showed a slight increase in FT of plants grown at 17°C, contrary to plants grown at 25°C. No significant changes in the levels of DWC were observed in the plants cultivated at 25 and 17°C.

All cultivars grown at 9°C showed significantly higher levels of WCS120 protein, sum of WCS120 proteins, FT and DWC, than did plants cultivated at 17°C. The highest FT (lowest LT_{50}), DWC and accumulation of WCS120 protein, and all WCS120 proteins were reached in those plants cultivated at 4°C. The differences in DWC and FT between cultivars increased with lowered temperature. On the other hand, a similar increase (the same differences) in the level of WCS120 proteins in all wheat cultivars were observed at 4°C, as well as at 9°C (Fig. 2).

It was possible to detect the differences between cultivars, according to the level of WCS120 protein or the sum of all WCS120 proteins from 17°C; while only LT50 or DWC from those at 9°C. Nevertheless, at 17°C, two groups of cultivars were distinguished: high frost tolerant M808 and Šárka; and medium or low tolerant Zdar and Bill, including spring Sandra, as well. At 9°C and 4°C three groups of cultivars were visible: high frost tolerant M808 and Šárka; medium or low tolerant Zdar and Bill; and

least tolerant spring Sandra (Fig. 1 and 2). These results indicate that WCS120 proteins started to accumulate early and at higher temperatures, in higher frost-tolerant wheat cultivars, even before any increase of FT. On the other hand, spring Sandra accumulated WCS120 proteins at a much lower temperature, and at significantly lower levels, then the other winter cultivars.

Dehydrins, LT50 and DWC in leaves developed at higher temperatures.

In order to know if it was possible to find differences in frost tolerances between cultivars (in leaves developed at a higher temperature, following a short cold-acclimation), two wheat and two barley cultivars were grown and hardened according to the second experimental procedure. The much more frost-tolerant winter wheat, MIR808 and the barley Luxor were compared with spring wheat Sandra and barley Atlas at 17°C, and then after 2 weeks at 9°C. In wheats, the most abundant WCS120 protein (and in barleys, the DHN5 protein (ortholog of WCS120)), were determined by the anti-dehydrin antibody (Fig. 3). In barley, DHN5 was the most abundant dehydrin detected on the gel blot (not shown).

In those plants grown only at 17°C a small level of DHN5 was detected in both barleys, while a higher level of WCS120 protein was observed only in winter wheat MIR808 (Figs. 3 and 4). After 2 weeks at 2°C, dehydrins accumulated in all cultivars; although at higher levels in winter cultivars, than in spring ones. The differences were larger between wheat cultivars in levels of WCS120, than between barley cultivars in levels of DHN5. This corresponded with FT, which was higher in winter, than in spring cultivars (Fig. 4).

The levels of FT, DWC, and the amount of dehydrins increased in the mature leaves after acclimation of plants at 9°C (Fig. 4). Surprisingly, the significant increases of DWC were only observed in the winter cultivars. However, barleys had a lower increase of DWC than wheats. The results show that cultivars grown at 9°C could be differentiated by levels of leaf FT, as well as by the accumulation of the dehydrins.

Discussion

In our experiments, the temperature-dependent changes in the level of FT, DWC, and the accumulation of dehydrins were compared in wheats and barleys. From dehydrins, we focused on those proteins which are cold-regulated in cereals; i.e., the WCS120

protein family in wheat, and the DHN5 protein (WCS120 ortholog) in barley (Sarhan *et al.* 1997).

We were able to distinguish and quantify all five of the most abundant members of the WCS120 protein family in wheat, and to compare them with the most abundant dehydrin (DHN5) in barley, utilizing protein gel blot analysis using anti-dehydrin antibody (Fig. 1, 3). Among the WCS120 proteins, the most abundant was WCS120, followed by WCS66; which corresponds to published data (Houde *et al.* 1995, Vítámvás *et al.* 2007). All of them rapidly accumulated after cultivation at lower temperatures (9 or 4°C). The sum of all WCS120 proteins had almost same dynamics as the WCS120 protein alone (Fig. 2); this was also shown by Vítámvás *et al*. (2007) who cultivated two winter wheats at 2°C. The fact that the frost tolerant cultivar Zdar did not accumulate any WCS66, demonstrates that the number of dehydrins is not fixed in wheat cultivars. The identification of the 26 kDa protein (found only in the Zdar) is currently in progress and we can, therefore, only speculate that this protein might be the product of a mutation in the *wcs66* gene.

From the protein blot analysis, it is clearly visible that the different frost tolerant wheat cultivars showed different threshold temperatures for the induction of the WCS120 proteins (Fig. 1, Fig. 3). It was possible to determine three groups of wheat cultivars according to the threshold temperature. Furthermore, the lower the temperature of cultivation, the higher the level and the faster the accumulation of WCS120 proteins were observed in the more tolerant cultivars, compared to low tolerant or sensitive ones. Vágújfalvi *et al.* (2000) and Galiba *et al.* (2001) found similar results for the accumulation of the COR14b protein, as well as for *cor14b* mRNAs in wheats. The threshold temperatures for the accumulation of DHN5 here, and for *corb14b* mRNA and COR14 proteins described by Crosatti *et al.* (1995, 1996), were confirmed in barleys. Finally, Zarka *et al.* (2003) showed, with gradual temperature downshift experiments in *Arabidopsis thaliana*, that lower temperatures resulted in a higher level of *Cbf* transcripts, and found that the threshold temperature, promoting the accumulation of *Cbf* mRNA, was 14°C. It is very probable that the entire cold-regulated pathway is both temperature and tolerance dependent, and that other COR proteins can also demonstrate similar dependences.

The association, mentioned above, between threshold induction temperature polymorphism and the cultivars' frost tolerances offer us the ability to use the studied COR proteins for the selection of both frost-tolerant and frost-sensitive genotypes. The

COR14 protein has been suggested for this purpose in barleys and wheats (Crosatti *et al.* 1996, Giorni *et al.* 1999, Vágújfalvi *et al.* 2000). Here, we compared two ortholog dehydrins, WCS120 in wheat and DHN5 in barley. Both were detected by using the same anti-dehydrin antibody. However, the wheats were already differentiated at a higher temperature (17°C); while the barleys only at a lower temperature (9°C). A similar conclusion, i.e., that the temperature threshold was higher in wheat than in barley, was published by Galiba *et al.* (2000) for the expression of *cor14b* genes. These results might be explained by a generally higher frost tolerance as well as a larger frost tolerance range in wheats than in barleys and/or by a higher sensitivity to lower temperatures of wheats than barleys (Prášil, Prášilová & Mařík 2007). However, the very low and similar levels of DHN5 in both barley cultivars at 17°C (Figs. 3 and 4) indicate that different mechanisms of COR protein induction between these two members of Triticae also can occur. This means that the wheat winter and spring cultivars were differentiated according to their threshold temperatures for the induction of expression (i.e., response: present or absent), as well as for accumulation (i.e., response: more or less) of the dehydrins. On the other hand, the barleys could only be differentiated according to the threshold temperature for accumulation of the dehydrin.

The role of dehydrins in plant responses to cold has recently been reviewed (Kosová *et al.* 2007), and it was postulated that these proteins protect other intracellular proteins and membranaceous structures against unfavourable structural changes caused by dehydration during cold. Moreover, for both the WCS120 and the DHN5 protein, information has been published that they exhibit a cryoprotective activity (Houde et al. 1995, Bravo et al. 2003). However, FT is a more complex trait, so that the accumulation of one type of compound alone could not explain the level of plant FT.

The higher levels of WCS120, DWC and FT in leaves developed at low temperature (9°C) for 5 weeks; contrary to those leaves developed at 17°C, and then exposed to 9°C for 2 weeks (compare Figs. 2 and 4). This could indicate two causes. First, the differences in the duration of cold hardening (i.e., the shorter time of hardening of the plants was insufficient for the higher levels of these traits). For instance, Prášil, Prášilová & Pánková (2004) had to harden plants of MIR808 at 3°C for 4 weeks to reach the maximum FT. The second possible cause of these differences could be explained by different physiological changes in the leaves developed at low temperature, contrary to leaves shifted to low temperature, as shown by Gray & Heath (2005) in the *Arabidopsis* metabolome.

The result, that highly frost tolerant wheat cultivars (M808 and Šárka) grown at 17°C accumulated a higher level of WCS120 proteins (although their level of FT and DWC were insignificant from low-tolerant cultivars), indicates that the threshold temperature for WSC120 proteins is higher than the temperature where it is possible to differentiate the wheats by a frost test. Cereals usually must be exposed to temperatures below 12°C (the lower, the better, i.e., they have to be hardened by cold) to evaluate their FT levels by frost tests (Fowler, Limin & Ritchie 1999, Prášil *et al.* 2007). Moreover, from Fig. 2 it is possible to see that the differences between cultivars in DWC and FT progressively increased with lowering of the growth temperature of plants, while differences in WCS120 protein(s) remained very similar. It seems that the level of WCS120 proteins increases proportionally (linearly); while DWC and FT rise more rapidly with the decreasing of growth temperature. Recently, we have observed that cold-re-accumulation of WCS120 proteins in winter wheat, following de-acclimation of plants, was lower than the re-established level of FT (Vítamvás & Prášil, unpublished). This means that cold regulation of the WCS120 proteins and FT can be different; however, the determination of FT in wheats using the LT_{50} values or the accumulation of WCS120 brought the same results for plants growing at constant low temperature (here at 9 or 4ºC).

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Figures

Figure 1. Accumulation of WCS120 proteins in the spring cultivar (Sandra) and the winter wheat cultivars cultivated at 25 (2 weeks), 17 (3 weeks), 9 (5 weeks) and 4 ºC (4 weeks, following 2 weeks at 17 ºC). A) Silver-stained 1D SDS-PAGE gel. B) Western blot with anti-dehydrin antibody (against the K-segment). The number indicates the growth temperature. Arrow indicates band visible only in Zdar (MW about 26 kDa). St-SDS-PAGE Standards, broad range.

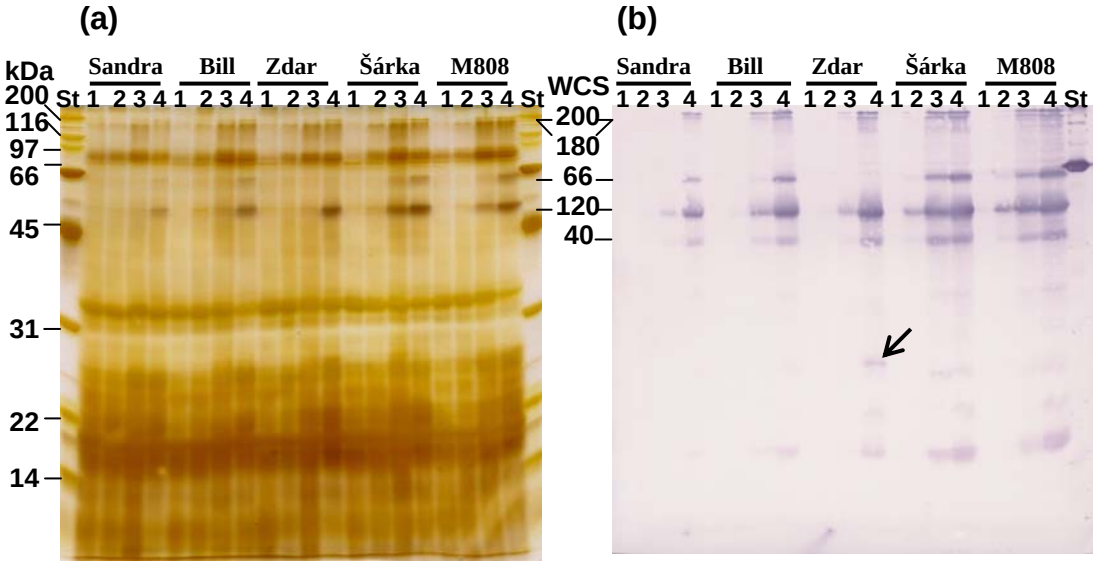

Figure 2. The differences in the LT_{50}, dry weight content (DWC), sum of the WCS120 proteins and amount of WCS120 protein (WCS120) in the wheats cultivated under different growth temperature (25, 17, 9, and 4 ºC; as in Fig. 1). Presented data are averages calculated from 3 repetitions at least. The highest value of integral density of sum of all WCS120 bands in one sample on W-blots was set to 100 % in each membrane and the averages were calculated from 4 independent repetitions (membranes). Vertical bars indicate $LSD_{0.05}$.

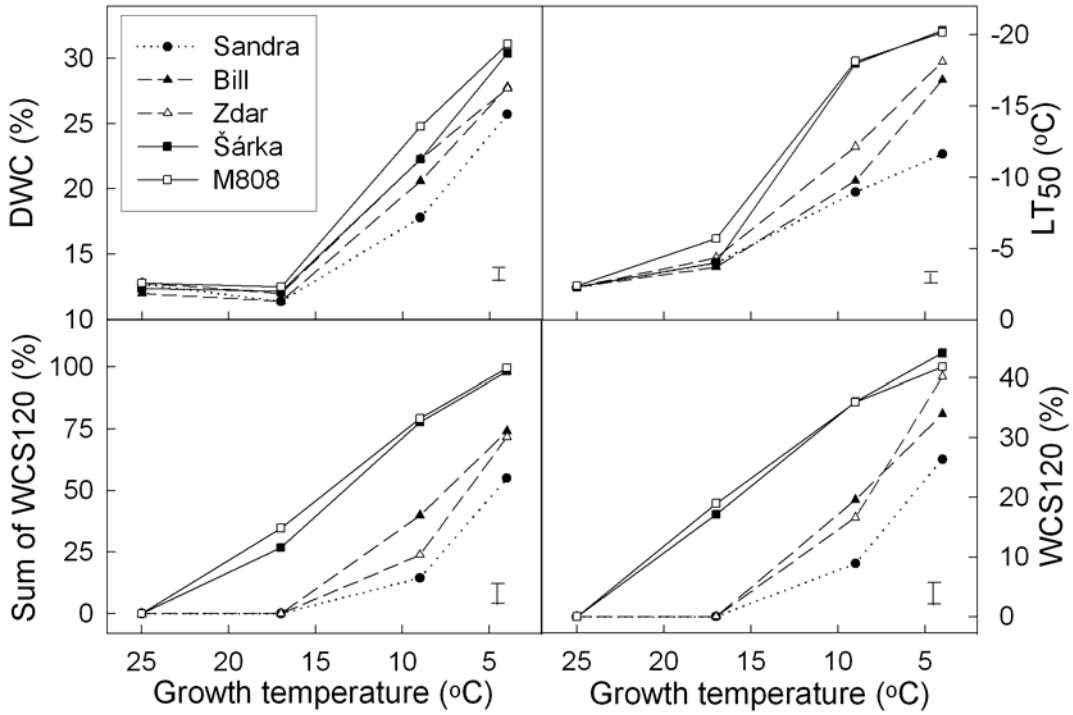

Figure 3. Comparison of DHN5 and WCS120 accumulation in the spring (A, Atlas 68; S, Sandra) and the winter (L, Luxor; M, M808) barley and wheat cultivars cultivated at 17 (3 weeks) and 9 ºC (2 weeks, following 3 weeks at 17 ºC). The number indicates the growth temperature.

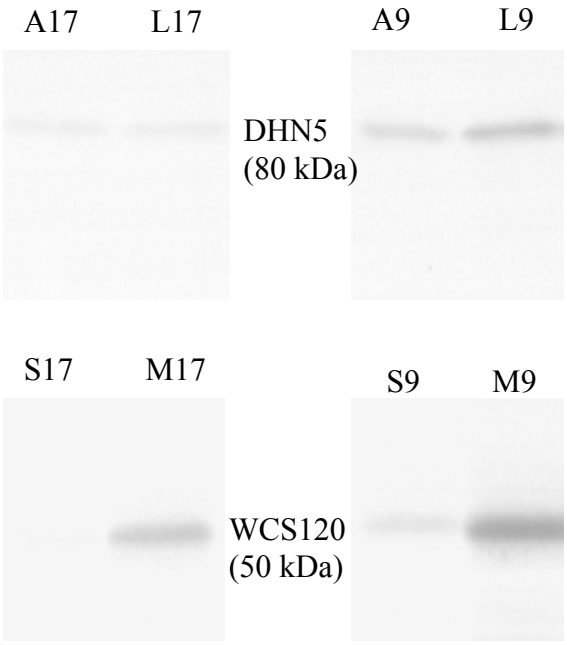

Figure 4. The differences in the amount of WCS120 or DHN5, dry weight content and LT_{50} in the leaves of the spring and winter wheat or barley cultivars cultivated at 17 and 9 ºC (as in Fig. 2). Presented data are averages calculated from 3 repetitions. The highest value of integral density of one band in the one sample on W-blots was set to 100 % in each membrane and the averages were calculated from 3 independent repetitions (membranes).