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REVIEW

The role of dehydrins in plant response to cold

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Abstract

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

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

Introduction

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

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

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

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

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

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

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

Cold stress and its physiological impacts on plants

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

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

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

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

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

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

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

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

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

The role of dehydrins in the cold response of herbaceous dicotyledons

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The role of dehydrins in the cold response of woody plants

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

The role of dehydrins in the cold response of cereals

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

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

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

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

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

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

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

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

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

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

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

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

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

Important regulatory mechanisms involved in dehydrin expression during cold

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

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

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

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

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

Relationships between *Vrn* genes, *Fr* genes (frost resistance genes which are located on homoeologous group 5 chromosomes) and the expression of CBF-regulated genes such as dehydrin genes are intensively studied in cereals (Danyluk *et al.* 2003, Kobayashi *et al.* 2005). The different regulation of *Vrn* gene expression could be one of the major causes of the different dynamics of FT development in spring and winter cultivars, involving the level of dehydrin expression and accumulation. A positive effect of dehydrin accumulation on the overwintering of young wheat plants was found by Stupnikova *et al.* (2002) in a frost-resistant winter wheat cv. Irkutskaia ozimaia under field conditions of eastern Siberia. A good correlation between dehydrin accumulation and FT was observed by Fowler *et al.* (2001) in the winter barley cv. Dicktoo during 10 weeks of cold acclimation (a time necessary for the fulfilment of vernalization requirement determined as the final leaf number). Significant differences in dehydrin accumulation between winter wheat cv. Norstar and spring wheat cv. Katepwa were observed by Fu *et al.* (2000) during 7 weeks of cold treatment. After 7 weeks of cold, dehydrins were nearly absent in Katepwa whereas in Norstar they accumulated to significant amounts. Similarly, differences in the accumulation of WCS120 proteins and FT between spring and winter wheat cultivars were observed by Stupnikova *et al.* (2001) after 9 d of LT-treatment (4 °C). However, significant differences in FT during a long-term cold acclimation were observed not only in winter *versus* spring cultivars, but also among various winter cultivars. The winter cultivars differing in their FT vary also in dehydrin content. Vítámvás *et al.* (2006) distinguished three-week-cold-acclimated winter wheat cvs. Mironovskaya 808 and Bezostaya 1 differing in their ability to develop FT on the basis of different accumulation of WCS120. Moreover, the winter cultivars with higher FT induced WCS120 proteins under higher temperature conditions (17 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 cv. Igri and spring barley cv. Atlas 68 during 16 weeks of cold acclimation. The cv. Atlas 68 showed a rapid increase in dehydrin accumulation during the beginning of cold treatment followed by a slow decrease in the rest of the treatment. Contrary to cv. Atlas 68, cv. Igri showed a slow increase in dehydrin accumulation at the beginning of cold treatment and the maximum of FT and dehydrin accumulation is reached later. We have found out that when the cold-acclimated plants reach their maximum FT, the amount of dehydrins decrease although cv. Igri can retain significant amount of dehydrins after 16 weeks of cold treatment (Kosová *et al.*, unpublished).

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

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

Methods of functional genomics used in cold stress research

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

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

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

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

Current conclusions and future perspectives

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

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

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

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

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

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

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