1. Introduction

1.1 Mechanism of freezing tolerance in plants

1.1.1 Acclimation capacity of plants

Cold, drought and salinity are those environmental stressors which affect plants in many respects and which, due to their wide-spread occurrence cause the most fatal economic losses in agriculture.

Plants differ in their capacity to cope with sub-optimal temperatures. The plants that often growing in tropical areas are injured at temperatures just below +10°C, while a low temperature tolerant plants can tolerate low, non-freezing temperatures, but are killed in temperatures a few degrees below zero.
Plants that can tolerate freezing temperatures employ two major strategies.
They either avoid freezing or tolerate extracellular freezing (Sakai and Larcher, 1987).
The characteristics of water limit the distribution of plants with freeze-avoidance strategy. Water molecules come together to form a stable ice nucleus, either spontaneously (homogeneous nucleation) or catalysed by ice nucleators (heterogeneous nucleation). Homogenous nucleation of pure water is – 38°C, at which it freezes spontaneously. Distribution of plants with freeze-avoidance strategy is thus limited to areas where temperatures below – 40°C are not encountered (Sakai and Larcher, 1987).
Perennial plants able to tolerate dehydration caused by extracellular ice formation are the coldest hardy ones and they can grow in the coldest areas of the earth.

1.1.2. Control of the freezing process

Freezing of the tissues is a controlled event in freezing tolerant species. Plants themselves can secrete heterogeneous nucleators to initiate ice formation in xylem vessels and in discrete regions where extracellular ice may cause minimal physical damage. These nucleators may contain proteinaceous or carbohydrate components as well as phospholipids or polysaccharides (Griffith and Antikainen, 1996). In addition, organic and inorganic debris, ice-nucleation-active bacteria (INA), other biological molecules and structures, and snow and sleet can act as heterogeneous nucleators (Pearce, 2001). Ice-nucleation sites in freezing tolerant plants have defined compositions, their amount may fluctuate seasonally and they are active in specific tissues, thus determining the temperature and the location at which the extracellular ice forms in plants (Griffith and Antikainen, 1996).

Antifreeze proteins (AFP) are other factors controlling freezing in plants, (Griffith and Yaish 2004) which associate with small ice-crystals, forming a hydrophobic coat (Antikainen *et al* 1993).
Some plant AFPs have been shown to be similar to pathogenesis related proteins, induced in plants in response to a pathogen attack (Griffith and Antikainen, 1996).
Ashworth and Pearce (2002) demonstrated that initial freezing is always extracellular, both in cold-acclimated and non-acclimated plants, as well as in plants that have no capacity to acclimate. Depending on woody plants species, they might show different strategies in different organs. In the cortex ice forms extracellularily, but xylem parenchyma exhibits either deep supercooling or extracellular freezing, depending on species (Pearce, 2001).

1.1.3. Injuries caused by sub-optimal temperatures

Injury caused in plants by low temperature may arise from various factors. In chilling sensitive plants injury is considered to be the consequence of physiological or metabolic dysfunction caused by the influence of low non-freezing temperatures on physiological processes. Plants that tolerate freezing temperatures by deep supercooling will die if the temperature decreases so low that their capacity for supercooling is exceeded and they freeze rapidly.
Tolerant plants that freeze extracellularily may be injured by cellular dehydration: The water potential of ice is lower than that of liquid water. Consequently, extracellular ice crystals grow by drawing water from the cells until the water potential of ice and cytosolic water are equal, thus dehydrating the cell contents. The water potential of ice is lower the lower the temperature is, hence cellular dehydration becomes progressively more severe when temperature falls, down to a limit set by vitrification. Membrane structures are damaged when the freeze-induced dehydration exceeds the dehydration tolerance of the cell (Steponkus, 1984). Injuries may also be caused indirectly. In trees cavitation of vessels in response to drought during winter may impair water movements later in the spring (Utsumi et al., 2003).

1.2 Protection against freezing

1.2.1. Protection of membranes against dehydration

Water is the driving force for the assembly of phospholipids into biological membranes in cells and, in part, for the conformation of proteins. Dehydration damage can be lethal when cells are not able to maintain their cellular organization, a situation leading to structural changes in membranes and protein denaturation (Hoekstra et al., 2001).

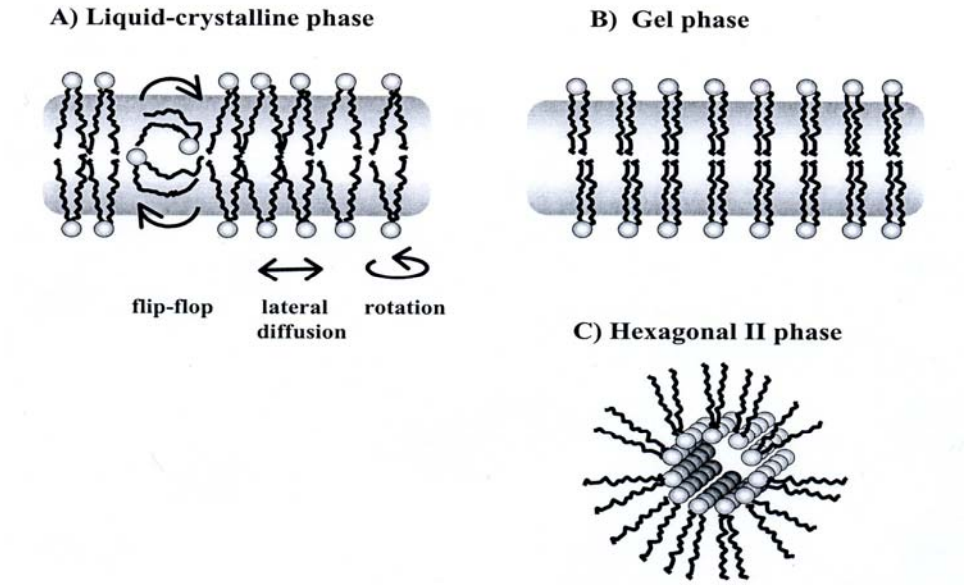

Fig. 1.1 Illustration of phospholipid layers in liquid-crystalline phase (A), in gel phase (B) and in hexagonal II phase (C).
In liquid-crystalline phase phospholipids have both lateral and kinetic motion whereas in gel phase lipids have less kinetic energy. In hexagonal II phase lipids are not in bilayer but they form long tubes surrounding water (Steponkus, 1984; Oliver et al., 2002; Hoekstra et al., 2001).

In fully hydrated cells, membranes are in a liquid-crystalline phase (Figure A), where lipids of the membrane have both lateral and kinetic motion. During dehydration water molecules are no longer helping to maintain the spacing between the phospholipid head groups, leading to a closer packing of the lipid molecules and an increase in the membrane phase transition temperature (*Tm*). This can result in phase transition of the membrane into the gel phase (Figure B) (Steponkus, 1984). With further dehydration, membranes undergo transition to hexagonal II phase in which membranes no longer form bilayers but three dimensional structures with long tubes of lipid surrounding water (Figure C). During rehydration membranes undergo new phase transitions, resulting in transient leakage of soluble cell contents trough membranes (Oliver et al., 2002). During mild drought tolerant plants accumulate compatible solutes and sugars. These are preferentially excluded from the surface of the proteins and membranes, thus forming a cohesive water layer around macromolecules and membranes, providing them a hydrated surrounding (Hoekstra et al., 2001).

Under more severe cellular dehydration, when water is almost absent, sugars are suggested to replace water in the hydration shell of the membranes, maintaining the spacing between phospholipid molecules and reducing the *Tm*. Sugars can form a carbohydrate glass with a high melting temperature. (Oliver et al., 2002).

1.3. Dehydrins (main characteristics)

The evolution of plants has resulted in the elaboration of protective mechanisms responsible for adaptation to constantly changing environmental conditions of growth. Stress induced changes in expression of certain genes represent the genetic basis for the development of the protective reactions. It is well known that intensity of growth and development of plants depends on their water supply. However, dehydration is a normal process during seed maturation, which is realized by programmed expression of specific genes. The phytohormone abscisic acid (ABA) plays a key role in the regulation of this process. Several genes specifically expressed in this ontogenetic period have been recognized. They include genes encoding LEA (Late Embryogenesis Abundant) proteins. LEA proteins have been found in ABA treated vegetating plants and also under the stress conditions that result in cellular dehydration such as drought, salinity, and low temperatures. One group of such genes encodes dehydrins (DHN), known also as group 2 late embryogenesis abundant (LEA) proteins. First dehydrins genes were isolated in late 1980's from cotton and rice (Baker et al., 1988; Mundy and Chua, 1988). Nowadays, genes encoding dehydrins have been cloned from numerous plant species belonging to such diverse groups as angiosperms, gymnosperms, mosses and lycopods (Svensson et al., 2002).
Detailed study of structural organization of dehydrins revealed the existence of repeated conservative domains providing specific spatial configuration that underlines dehydrin involvement in the reactions preventing loss of water. Dehydrins are widely distributed in plants. They significantly vary in amino acid composition and by molecular mass (from 100 to 600 amino acid residues). The amino acid composition of these proteins is characterized by high content of charged and polar residues, and this determines their biochemical properties including thermostability. This may promote their specific protective functions under conditions of cell dehydration: dehydrins may prevent coagulation of macromolecules and maintain integrity of crucial cell structures.

1.3.1. Structure of dehydrins

Dehydrins are characterized by highly conserved sequence motifs denominated as K, S, and Y segments (Close, 1996). K segment is obligate for all dehydrins.
By definition, dehydrins contain a lysine rich domain called the K-segment, (EKKGIME/DKIKELPG), which is repeated up to 15 times and is often located in the C-terminal part of the protein (Close, 1996). Computer aided analysis of amino acid sequences of K segments demonstrated the possibility of formation of amphipathic α-helix (Baker and Steele, 1988). Circular dichroism (CD) data also support formation of secondary α-helical structure by the K segments. For example, the CD spectrum of vigna dehydrin suggests that in the presence of 10% SDS up to 15% of amino acids are involved in formation of secondary α-helical structure (Ismail and Close, 1999). Similar results have been obtained for

maize dehydrin G50 (Ceccardi et al., 1994). K-segments may form amphipathic α -helices (Dure et al., 1989; Close, 1996). It has been shown that in the presence of SDS, dehydrins can form α -helical structures, suggesting that dehydrins may *in vivo* fold into a more ordered structure by interacting with other molecules or membranes (Ismail et al., 1999; Hara et al., 2001). Recently, Koag et al. (2003) demonstrated that binding of maize DHN1 to lipid vesicles was associated with an increase in α -helicity of the protein.

Many dehydrins contain an S-segment that consists of serine residues; this site may be phosphorylated. The latter was demonstrated for maize RAB17 and tomato TAS14 dehydrins (Campbell et al., 1998). Phosphorylation of S-segment is suggested to promote dehydrin interaction with specific signal peptides followed by their translocation into the nucleus (Close et al., 1997). Phosphorylation of dehydrins has been shown to be related to their ability to bind calcium (Heyen et al., 2002). Dehydrins can also be glycolysated (Golan-Goldhirsh, 1998; Levi et al., 1999).

The N terminal region of many dehydrins contains another conserved sequence, the Y-segment. This sequence, (V/T)DEYGNP, shares significant homology with the nucleotide binding site of plant and bacterial chaperones; however, binding of dehydrin Y-segments with nucleotides remains to be well documented (Close et al., 1997).

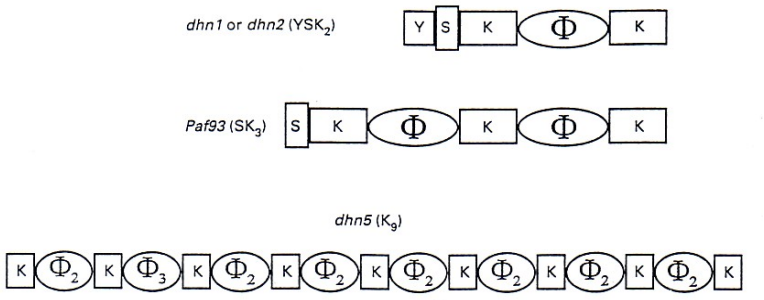

Fig. 1.2. Schematic representations of several barley dehydrins. (Close et al., 1997) Three types of barley dehydrins are shown that display various combinations of conserved motifs. In each example, K-segment are present but the number of them varies from two to nine.

Besides these three conserved segments, dehydrins also contain less conserved regions enriched with glycine and polar amino acid residues. These regions were named Φ -segments (Close, 1997; Ismail et al., 1999).
In some dehydrins Φ-segments are localized as single copies between K-segments; they are enriched with proline and alanine residues (e.g., *Arabidopsis thaliana* COR47 protein (Close, 1997).

Dehydrin proteins are characterized by a wide range of molecular masses from 9 to 200 kDa (Close et al., 1997). Dehydrins contain a large amount of glycine and

charged and polar residues, making them highly hydrophilic. This may partly explain their characteristic boiling stability.

1.3.2. Classification of dehydrins

By using the numbers of Y, S and K-segments in dehydrins, it is possible to classify them in sub-classes and five distinct types of dehydrins have been found in higher plants: YnSKn, SKn, Kn, YnKn, and KnS (Campbell and Close, 1997). It has been suggested that if the different YSK structural types have a distinct function, all plant species could in principle have at least one of the each type of dehydrins (Svensson et al., 2002).

Subdivision of dehydrins into several classes depends on their structural organization and biochemical properties (Allagulova *et al* 2003).
Though the precise function of dehydrins has not been established yet, much recent *in vitro* data clearly indicates that dehydrins belonging to different subclasses exhibit distinct functions.

In vitro experiments revealed that YSKn type dehydrin bind to lipid vesicles that contain acidic phospholipids (Nylander et al., 2001), while others (KnS-type) were shown to bind metals (Bomal et al., 2002), scavenge hydroxyl radicals (Hellwege et al., 1994) or display cryoprotective activity towards freezing-sensitive enzymes (Adams et al., 2000; Busk et al., 1998). Two other types, the SKn and K-type, seem to be mainly involved in cold acclimatization and drought resistance.

1.3.3. Structure of *Arabidopsis* dehydrins

RAB18 (Y2SK2), LTI29 (SK3), ERD14 (SK2), LTI30 (K6) and COR47 (Svensson et al., 2000) have been expressed in recombinant *E. coli* cells. Isolation and purification of native *Arabidopsis thaliana* dehydrins allowed the investigation of their biochemical properties *in vitro*. Generally, Arabidopsis dehydrins are enriched with glycine and lysine residues, but they lack cysteine and tryptophan (Wisniewski et al., 1999).
For example in Arabidopsis LTI30 dehydrin with molecular mass of 21 kDa, glycine, histidine, lysine, and threonine represent 56% of the total amino acids, whereas cysteine, tryptophan, arginine, and valine were not found (Svensson et al., 2000), this explains the highly hydrophilic nature of this protein. Dehydrins are not very likely to form oligomers and they are intrinsically unstructured proteins (Ceccardi et al., 1994; Ismail et al., 1999; Hara et al., 2001).

1.3.4. Functional studies of dehydrins

The universal occurrence of dehydrins in any studied plant species and their accumulation in response to various stresses support their participation in abiotic stress adaptation in plants. The defined mechanism of action of dehydrins is still unresolved.

The wide distribution of dehydrins in the vegetative tissues of plants grown under normal conditions suggests that these proteins may also play an essential role during plant growth. The precise function each of dehydrin type *in planta* has not been established. To get more insight in the potential roles dehydrins play in plants, their cryoprotective properties, ability to bind lipids and metals, and antioxidative activity have been thoroughly analyzed in *in vitro* studies.
Based on their hydrophilic structure dehydrins can act as compatible solutes under mild water stress, and under more severe cellular dehydration, when water is almost absent. Dehydrins can interact with other proteins with their hydroxylated residues stabilizing structures of macromolecules and membranes. Thus, dehydrins could be solubilizing agents with detergent and chaperone activities (Close, 1996).
Dehydrins have also been shown to have in vitro cryoprotective (Close, 1996; Rinne et al., 1999; Wisniewski et al., 1999) and antifreeze (Wisniewski et al., 1999) activity in several plant species, confirming participation of dehydrins in cold acclimation of plants. The proper function of dehydrins during cellular dehydration might involve complex processes involving the concerted action of group of dehydrins or interaction with other protective molecules such as other LEA proteins or compatible solutes (Hoekstra et al., 2001).

In pure form dehydrins are thought to be intrinsically unstructured (Lisse et al., 1996), but they may form intrinsic structures when bound to target molecules (Garay-Arroyo et al., 2000). The K-segment present in all dehydrins resembles a lipid-binding class A2 amphipathic α helical segment found in apolipoproteins and α synucleins (Davidson et al., 1998). The presence of K-segment raises the question of whether dehydrins bind lipids, bilayers or phospholipid vesicles. If the K-segment forms an α-helical structure similar to that of the A2 amphipathic segment, one of its roles would be hydrophobic interactions with membranes and denatured proteins (Close, 1997). It has been hypothesized that dehydrins function as surfactant molecules, acting synergistically with compatible solutes to prevent coagulation of colloids and a range of macromolecules (Close, 1997). However, direct evidence for such function *in planta* has not been established. There is evidence from *in vitro* experiments that dehydrins have a propensity to engage *in vitro* hydrophobic interactions that may involve the formation of amphipathic α -helices by the K-segment. More direct evidence for the capability of dehydrins to bind lipids was provided by studies on maize (*Zea mays*) DHN1 dehydrin (RAB17; YSK2-type), isolated from mature seeds (Koag et al., 2003) As

shown, DHN1 binds *in vitro* to lipid vesicles that contain acidic phospholipids, and this binding was found to be more favorable to vesicles of smaller diameter (SUV) prepared from negatively charged phospholipids containing phosphatidic acid (PA), phosphatidyl-Ser (PS) and phosphatidyglycerol (PG) (Koag et al., 2003).

The association of DHN1 with PA-derived vesicles results in an apparent increase in the α-helicity of the protein by 9%, similar to that observed in the presence of 10 mM SDS. The increase in the α -helicity of maize DHN1 when bound to phospholipid vesicles *in vitro* may suggest that the DHN1 also takes on α -helical structures when associated with vesicles *in vivo*, and that the two K-segments present in the protein are involved in membrane binding. Those authors concluded that dehydrins, and presumably similar plant stress proteins abundant in late embryogenesis, may undergo function-related conformational changes at the water-membrane interface, perhaps related to the stabilization of vesicles or other endomembrane structures under stress conditions (Koag et al., 2003).
Putative functions of dehydrins also include involvement in detoxification of salt ion excess observed during cell dehydration. It was shown that RAB18, LTI29, LTI30, and COR47 dehydrins from *Arabidopsis thaliana* can interact with Cu^{2+} and Ni^{2+}. The dehydrin LTI30 can also bind Cu^{2+} and Zn^{2+}; however, it should be noted that these dehydrins lack any specific metal-binding motifs.
Metal ion binding capacity of *Arabidopsis thaliana* dehydrins may be attributed to the presence of a high number of histidine residues (Wisniewski et al., 1999)

1.3.5. Dehydrins and cryoprotective activity

It was recently reported that some dehydrins display cryoprotective activity towards freezing-sensitive enzymes. The *Citrus unshiu* CuCOR19 dehydrin was shown to protect catalase and lactate dehydrogenase against freezing inactivation, and it was more effective than compatible solutes such as sucrose, glycine betaine and proline, or BSA (Hara et al., 2001). Analysis of the circular dichroism spectrum of CuCOR19 showed that the major secondary structure of CuCOR19 in the solution is a random coil. It is likely that the random coil structure of dehydrins may play an important role in the cryoprotection of freezing-sensitive enzymes. Low temperature reduces the activity of oligomeric proteins by the dissociation of subunits. Randomly coiled motives of CuCOR19 could make cohesive layers with the surface of the oligomers, and prevent the dissociation of the active forms. Functional analyses of cryoprotective and antifreeze activity have been also reported for *Prunus persica* PCA60 dehydrin (YK) (Wisniewski et al., 1999) and the *Betula pubescens* one (Rinne et al., 1999). PCA60 preserved the *in vitro* enzymatic activity of lactate dehydrogenase after several freeze-thaw cycles in liquid nitrogen. PCA also has shown distinct antifreeze activity as evidenced by ice crystal morphology and thermal hysteresis (Wisniewski et al., 1999). Dehydrins from *Betula pubescens* were shown to

enhance α-amylase activity in the presence of polyethylene glycol (Rinne et al., 1999). These *in vitro* tests suggest that some dehydrins may protect enzymes under low temperature stress *in vivo*.

Many studies reported a positive correlation between the accumulation of dehydrin transcripts or proteins and the tolerance to freezing, drought, and salinity (Rodriguez et al., 2005; Nylander et al., 2001; Houde et al., 1995).

Puhakainen *et al*.(Puhakainen et al., 2004) provided the data that overexpression of multiple *Arabidopsis* dehydrins genes such as *LTI29* (*ERD10*, SK3-type) and *LTI30* (K6) resulted in increased freezing tolerance and improved survival under exposure to low temperatures, demonstrating that dehydrins do contribute to freezing tolerance. In another side overexpression or antisense inhibition of the RAB18 (Y2SK2) gene had no effect on freezing tolerance in A*rabidopsis* (Lang and Palva, 1992).

1.3.6. Location of Dehydrins in plant tissues

Many studies demonstrated that dehydrins are distributed in different tissues during plant growth and development. Some dehydrins are mainly found in mature seeds. RAB18 (Y2SK2-type) from *Arabidopsis* is localized in all the parts of the embryo and in the endosperm of mature seeds (Goday et al., 1994) *Arabidopsis* ERD14 (SK2) and ERD10 (LTI29, SK3-type) have been shown to localize in root tips, and in the vascular tissues of roots, stems, leaves and flowers (Nylander et al., 2001).
Some dehydrins exhibit a localization specific to cell types, e.g. pollen sacs, guard cells, root meristematic cells (Nylander et al., 2001) or plasmodesmata (Karlson et al., 2003).
RAB18 (Y2SK2) from *Arabidopsis* showed accumulation specific to the stomatal guard cells of stems, leaves and flowers (Nylander et al., 2001).
From all these studies, it can be concluded that there are different types of dehydrins that can localize in the same tissues during normal growth and that most of them are to be found in vascular tissues and surrounding cells.

In plants subjected to stress leading to cellular dehydration, such as drought, low temperature and salinity, there was a substantial increase in the content of most dehydrins, and they also appeared in other tissues than under normal growth conditions. For example, the *Arabidopsis* ERD14 and ERD10 (LTI29) dehydrins, which were primarily localized in root tips and in the vascular tissues of roots, stems, leaves and flowers in plants grown under normal growth conditions, were detected in cells of all the tissues in cold stressed plants (Nylander et al., 2001).

Similar data was obtained for the distribution of DHN24 from *Solanum sogarandinum* (Rorat et al., 2006) and P-80 from *Hordeum vulgare* (Bravo et al., 1999) in cold acclimated plants.

Other dehydrins, such as LTI30 (K6) from *Arabidopsis*, were not detected in plants grown in control conditions, but did accumulate in all the tissues of the roots, in the vascular tissues of stems, leaves and flowers, and in the pollen sacs under cold conditions (Nylander et al., 2001).

Unlike the dehydrins mentioned above, the *Arabidopsis* stomatal guard cell RAB18 dehydrin (Y2SK2) was not induced by low temperature, but it was strongly induced by exogenous ABA. A high elevation was also observed in the ERD14 content in plants subjected to ABA and NaCl treatments (Nylander et al., 2001).
This data clearly shows that dehydrins belonging to different sub-classes may accumulate in the same organs or tissues in plants grown under normal conditions, and that their amounts substantially increase under the cell dehydration conditions that occur during seed maturation or in vegetative tissues subjected to environmental stress such as drought, low temperature and elevated salinity.

1.3.7. Subcellular location

Subcellular localization data revealed that dehydrins are localized in various cell compartments including the cytosol, nucleus, vicinity of the plasma membrane, mitochondria and vacuole (Houde et al., 1995; Danyluk et al., 1998; Rorat et al., 2004; Heyen et al., 2002). However, they are primarily localized in the cytoplasm and nucleus.

Specific functions of dehydrins may be attributed to their particular localization in the cell. Dehydrins are localized in cytoplasm and nucleus (Solomon et al., 2000; Campbel and Close, 1997). This was clearly demonstrated for wheat WCS120, maize RAB17, and tomato TAS14 dehydrins (Sarhan et al., 1997; Goday et al., 1994), peach PCA60 dehydrin (Wisniewski et al., 1999), and birch 24 kD dehydrin (Rinne et al., 1999).

Immunomicroscopic studies revealed that treatment of maize seeds with ABA for 15 min resulted in dehydrin accumulation in cytoplasm; during prolonged treatment they were detected in nucleus, and the maximal increase in dehydrin level in the nucleus and cytoplasm was observed after 48 h incubation with ABA (Egerton et al., 1997).
In the nucleus dehydrins were associated with heterochromatin, euchromatin, nucleoplasm and nucleoli; in cytoplasm they were found mainly in cytosol, some quantity of dehydrins was also detected in organelles (Egerton et al., 1997).

Nuclear localization of dehydrins in plant cells is associated with their protective role in stabilization of transcription machinery to unfavorable conditions.

For example, pea embryo p16 dehydrin with molecular mass of 16 kD was isolated from the fraction of histone H3; this suggests involvement of this dehydrin in mechanisms responsible for structural integrity of chromatin during cell desiccation in the process of seed formation (Castillo et al., 2002).

Mechanisms underlying dehydrin translocation into the nucleus are not perfectly understood, but certain evidence exists that S-segment is involved in this process. It should be noted that not all dehydrins have been found in the nucleus. Dehydrins can be associated not only with plasmalemma, but also with intracellular membrane structures (as shown for parenchymal cells of maize scutellum (Egerton et al., 1997) and onion epidermal cells (Close, 1997).

Membrane localization of dehydrins may suggest their involvement in stabilization of cell membrane structures; this is especially important under unfavorable (environmental) conditions (Close, 1996; Close, 1997).

To summarize the data presented above, the following conclusions may be drawn:

- Dehydrins belonging to group 2 of the LEA, the so-called dehydrins, are widely distributed in the plants.
- On the basis of amino acid sequence similarity and structural characteristics, dehydrins can be divided into different types.
- Dehydrins can be localized in different vegetative tissues during growth under normal conditions, and are substantially accumulated in the cells of all tissues under conditions leading to cell dehydration, such as drought, low temperature and salinity, and during natural dehydration processes that occur during seed maturation and in dry seeds.
- There is no correlation between dehydrin type and tissue localization.
- Several dehydrin proteins types may be localized in the same tissue.
- The precise function of dehydrins *in planta* has not been established but *in vitro* findings revealed that each dehydrin type could have a specific function.

Accumulation of dehydrins in vegetating plants in response to cold temperatures, salinity, and moisture deficit attracts much interest in relation to possible protective functions of dehydrins under conditions of cell dehydration.

The aim of this work is to analyze potential functions of four dehydrins from Arabidopsis thaliana (LTI 29, ERD14, COR47 and RAB18) and mechanism of their activity in vitro.
Because of that, it was necessary to affirm which of analyzed proteins have cryoprotective activity and in which percentage.
With a view to determine mechanism of cryoprotective activity it was important to analyze binding ability of proteins to membrane (thylakoid membrane) and is that cryoprotective activity in correlation to binding activity of analysed proteins.

Seizing Boyle van't Hoff type of analyses we deemed necessary to examine whether presence of analyzed proteins can change membrane permeability, which can be essential for defining mechanism of cryoprotective activity.
Also using of light scattering technique we affirmed that presence of Ca^{2+} and Mn^{2+} ions can have influence on membrane permeability and activity of cryoprotective proteins.

1.4. Cryoprotectin a plant lipid-transfer protein stabilizes membranes during freezing

The existence of proteins that can protect a biological membrane against freeze-thaw damage was first reported by Heber and Kempfle (1970). They isolated a protein fraction from cold acclimated spinach and cabbage leaves that prevented the inactivation of cyclic photophosphorylation in spinach thylakoid membranes during freeze-thaw cycle to -25˚C. Later was demonstrated that this cryoprotective activity is not due to non-specific effects caused by the presence of soluble proteins. In addition, treatment of the extracts with a protease confirmed that the cryoprotective activity is indeed due to the presence of protein and not to the presence of contaminants such as sugars (Hincha et al., 1990; Hincha and Schmitt, 1992). These authors have purified a cryoprotective protein from cold-acclimated cabbage leaves (*Brassica oleracea*) to electrophoretical homogeneity and called this protein cryoprotectin (Sieg et al., 1996). Partial sequencing of cryoprotectin showed that analyzed protein belongs to LTP (lipid transfer protein) family (Sieg et al., 1996). Further investigations have shown that cryoprotectin has no lipid transfer activity like other known LTPs.
Cryoprotectin is also characterized by high stability at high temperatures and in the presence of chemical denaturants (Watanabe et al., 1992; Hincha et al., 2001). This has been attributed to the stabilizing influence of four disulphide bridges characteristic for LTPs as small proteins (Lindorff-Larsen and Winther, 2003).

The apparent molecular mass of cryoprotectin as determined on western blots was close to 10 kDa, whereas in silver-stained gels the apparent molecular mass was closer to 7 kDa (Sieg et al., 1996). This discrepancy is most likely due to the use of different sets of marker proteins. The cryoprotective activity of cryoprotectin was strictly dependent on Ca^{2+} and Mn^{2+} and could be completely inhibited with EDTA (Hincha et al., 2001). It was also shown (Sror et al., 2003) that cryoprotectin binds to thylakoid membranes and that this binding is necessary for cryoprotection.

Mechanism of cryoprotective activity of cryoprotectin *in vitro* was investigated by measuring thylakoid volume (thylakoids isolated from the leaves of nonacclimated spinach (*Spinacia oleracea*)). Thylakoid volume is plotted as a function of reciprocal osmolallity of the sucrose solutions used to suspend the membranes (Boyle–van't Hoff plot). Based on this studies authors conclude that

cryoprotectin reduces solute permeability of the membrane, leading to less solute loading of the membrane vesicles during freezing and therefore to less osmotic rupture during thawing (Hincha et al., 1999).

In this study cryoprotectin from cabbage (*Brassica oleracea*) was used as a reference system (positive control) according to cryoprotective activity.