Functional Studies of the Role of Plant Dehydrins in Tolerance to Salinity, Desiccation and Low Temperature

Jan Svensson
Department of Plant Biology
Uppsala

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Abstract

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Dehydrins are a group of proteins believed to play a fundamental role in plant response and adaptation to abiotic stresses that lead to cellular dehydration. This group of proteins can be found in seeds in the later maturation stage and the desiccated seed, and are induced by salt, drought and low temperature stress. Dehydrins share no sequence homology with any known protein or enzyme and have so far only been found in photosynthesizing organisms.

We have purified four recombinant dehydrins (RAB18, LTI29, LTI30 and COR47) and introduce a method previously not used for purification of dehydrins; immobilized metal ion affinity chromatography. Furthermore the accumulation of both transcript and protein was analyzed for five dehydrins (ERD14, RAB18, LTI29, LTI30 and COR47). Based on the accumulation data the dehydrins could be divided into four groups: (I) LTI30 cold specific, (II) RAB18 ABA regulated, (III) ERD14 constitutive and (IIII) LTI29 and COR47 mainly cold induced. Immunolocalization studies of four dehydrins (ERD14, LTI29, LTI30 and RAB18) showed tissue and cell type specific accumulation in unstressed plants. ERD14 and LTI29 were detected in the root tip and the vascular tissue whereas RAB18 was detected in stomatal guard cells. LTI30 was not detected in unstressed plants. In stress treated plants RAB18, LTI29 and ERD14 were detected in most cells whereas LTI30 was only detected in the vascular tissue and in pollen sacks. Ectopic expression of multiple dehydrins in Arabidopsis resulted in improved survival of plants exposed to drought and freezing stress. In addition, salt stressed seeds overproducing DHNs showed improved germination/cotyledon emergence. We isolated a dehydrin gene (*DHNA*) from *Physcomitrella patens* which shared some characteristics of dehydrins cloned in higher plants. *DHNA* was found to be expressed during salt and water stress and by ABA treatment.

Keywords: dehydrins, low temperature, drought, ABA, salinity, protein purification, Arabidopsis, *Physcomitrella patens*

Author's address: Jan Svensson, Department of Plant Biology, Swedish University of Agricultural Sciences, P.O. Box 7080, SE-750 07 Uppsala, Sweden. E-mail: Jan.Svensson@vbiol.slu.se

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Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. Svensson, J., Palva, E. T. and Welin, B. 2000.
Purification of Recombinant *Arabidopsis thaliana* Dehydrins by Metal Ion Affinity Chromatography. Prot. Exp. and Purif. 20: 169-178.

II. Nylander, M., Svensson, J., Palva, E. T. and Welin, B. 2001.
Stress-induced accumulation and tissue specific localization of Dehydrins in *Arabidopsis thaliana*. Plant Mol. Biol. In press.

III. Puhakainen, T., Svensson, J., Mäkelä, P., Niklander-Teeri, V., Heino, P. and Palva, E. T. Ectopic expression of dehydrin genes enhances tolerance to freezing and drought stress in Arabidopsis. Manuscript.

IV. Svensson, J. and Welin, B. Cloning and Characterization of a drought- and salt-induced dehydrin-like gene in *Physcomitrella patens.*
Manuscript.

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Abbreviations

ABA	abscisic acid
AOS	active oxygen species
DHN	dehydrin
GST	glutathione-S-transferase
GUS	β-glucuronidase
IMAC	immobilized metal ion affinity chromatography
LEA	late embryogenesis-abundant
MW	molecular weight
PAGE	polyacrylamide gel electrophoresis
RAB	responsive to ABA
SDS	sodium dodecyl sulphate

Introduction

Plants are constantly exposed to a variety of environmental stresses causing reduced crop yields. The annual economic losses caused by freezing alone is estimated at US $ 14 billion worldwide and the estimated yield of field grown crop plants is only about 20% percent of their full genetic potential (Boyer, 1982; Steponkus *et al.*, 1993). As a result, great interest has been focused on plant response to abiotic stresses, particularly desiccation, salinity and low temperature.

Drought, salinity and freezing are stresses which all lead to cellular dehydration through different mechanisms. This common component of water stress is evident in shared molecular responses to these stresses in many plant species. Examples of such responses are common genes induced by all three types of stress and the importance played by the phytohormone abscisic acid (ABA). Moreover, studies of different plant species with different tolerances to the above mentioned stresses has led to the hypothesis that the genetic information for tolerance exists in all higher plant (Michel *et al.*, 1993). The tolerance in a stress-sensitive plant is however only expressed in desiccated seeds and pollen grains.

In my work I have focused on one such common theme in plant response to abiotic stress, the accumulation of a group of proteins, the dehydrins (DHNs). DHNs are thought to play an important general protective role during cellular dehydration in all plant tissue. This assumption is however based more on gene expression and protein accumulation data in dehydrated plant tissue than on more direct evidence for such a role. I hope that my results presented in this thesis will shed more light on the protective function played by this large protein family during abiotic stress in plants.

Water deficit stress

Water is the central molecule for all forms of life. It accounts for 85-95% of the weight of most plants and 5-10% of the weight of seeds. All chemical reactions in plants occur in water based solutions. Nutrient transport, plant growth and photosynthesis are all dependent on water.

The movement of water through plants is determined by differences in water potential (Ψ). Water always flows passively from areas of high water potential to areas of low water potential and the movement of water into, through and out of plants is regulated by the water potential. Pure water has a potential of zero, measured in units of pressure, pascals (Pa). The water potential in a system is dependent on three factors; pressure potential Ψ_p, solute (osmotic) potential Ψ_s and the matrix potential Ψ_m. Consequently the water potential can be modified by solutes, physical pressure and by adhesion to matrices. A good rule of thumb for water movement is; water moves from an area of high purity to an area of low

purity. Water uptake is a passive process. Under conditions of transpiration, water moves passively through the root in response to a water potential gradient set up by transpiration, the so called transpiration-cohesion hypothesis. Transpiration is controlled by stomata (guard cells) and is important for, cooling (liquid to vapor), gas exchange and water movement. Water moves through root tissues to the xylem and is transported to the canopy. Lateral movement of water goes via apoplastic, symplastic and transcellular pathways. From the xylem, water is transported to the vascular bundles in leaves and finally reaches stomata and evaporates, thus contributing to the water potential necessary for water uptake.

Water deficit is mainly caused by three environmental stresses; salinity, low temperature (freezing) and drought.

Salinity

High salt concentration excerts two stresses on the plant cell, an ionic stress and an osmotic stress. The ionic stress is mainly caused by high Na^+ and Cl^- concentrations, which leads to an influx of ions into the plant cell and an altered ion homeostasis. The osmotic stress is caused by lowering of the soil water potential leading to a reduced water uptake and eventually to cellular dehydration.

Salt tolerant plants (halophytes) have adopted several modes of tolerance to high salt concentrations: exclusion of Na^+ uptake, compartmentalization of Na^+ in the vacuole and active excretion through special salt glands (Casas *et al.*, 1991; Popp, 1995; Thomas and Bohnert, 1993). Halophytes are dependent on the avoidance of salt in the cytoplasm and are not tolerant to salt at the molecular level. The only organisms truly tolerant of salinity at the molecular level are halobacteria.

Low temperature (freezing)

There are two types of low temperature stress; chilling stress for non-freezing temperatures and freezing stress at temperatures below 0°C. During freezing stress, ice can be formed both in the intracellular and extracellular space. If ice is formed intracellularly it is always lethal to the plant cell due to disruption of the cell structure. Tolerance to freezing is therefore dependent on extracellular ice formation (Levitt, 1980; Sakai and Larcher, 1987). At cooling rates below 2°C per hour, which is normally the case in nature, extracellular ice is formed. Ice nucleators and the lower solute concentration of the water in the extracellular space also promote this ice formation. When ice forms, water moves from within the cell to the growing ice crystal outside because the water potential of solid water (ice) is lower than that of liquid water (Levitt, 1980). Hence freezing leads to cellular dehydration similar to both salt (osmotic) and drought stress.

Plants have two main strategies to overcome freezing stress; avoidance or tolerance (Levitt, 1980). Avoidance of freezing is generally achieved by supercooling (undercooling) or absence of freezable water (Levitt, 1980; Sakai and Larcher, 1987). Supercooling is achieved by small cell size, little space for

nucleation, low moisture content, absence of internal nucleators, barriers against external nucleators and the presence of anti-nucleator substances (Levitt, 1980). Tolerance to freezing involves a process called cold acclimation (hardening). The triggers for cold acclimation in woody plants are low non-freezing temperatures and shortened day length, whereas annual plants only require low non-freezing temperatures. The degree of hardening differs between different species, many trees can increase their tolerance to -50°C and overwintering crop plants can survive temperatures as low as -30°C whereas annual plants can survive to -10°C (Levitt, 1980; Sakai and Larcher, 1987).

Drought

Water availability is one of the most important factors for plant growth. Any decrease in water availability has an immediate and serious effect on growth, and on processes ranging from photosynthesis to solute transport and accumulation. During periods of low water availability, stomata close to limit water loss. Since stomata are the means of CO_2 entry, this affects CO_2 intake and assimilation and therefore plant growth. Drought stress is a more severe dehydrative stress than freeze-induced dehydration. While freezing cannot remove the non-freezable water from proteins, membranes and phospholipids, drought can (Crowe *et al.*, 1990).

Plants have evolved several adaptations to avoid drought, such as small and thick leaves, leaf angle, spines and waxy coats to minimize water loss through evaporation. Metabolic adjustment to water stress is evident in crassulacean acid metabolism (CAM) and C_4 photosynthetic pathways. CAM plants close their stomata during daytime and combined with dark fixation of CO_2, this reduces water loss without limiting photosynthesis. C_4 plants exhibit no photorespiration and can fix CO_2 even in hot, dry conditions, requiring less water as a consequence. Although these mechanisms allow plants to lessen the severity of drought stress they do not make the plants tolerant of desiccation. One group of plants that are desiccation tolerant are the resurrection plants (poikilohydric). *Craterostigma plantagineum* has adapted to arid environments and can survive the loss of up to 95% of its water content (Scott, 2000). Resurrection plants survive desiccation by entering a quiescent stage and upon rehydration rapidly revive and are restored to their former state (Scott, 2000).

Seed development

Water deficit is a normal component in seed development, common to many higher plants. During seed maturation, as much as 90% of the original water is removed when the seed attains a state of dormancy (Leprince *et al.*, 1993). Seeds are not able to withstand desiccation at all developmental stages but only within a few days during late maturation prior to drying (Aldridge and Probert, 1992). During the later stages of maturation, a highly abundant set of proteins

accumulate; the late embryogenesis abundant (LEA) proteins. There are several different classes of LEA proteins of which DHNs constitute one (LEA II).

Stress injury and cellular response

A multitude of injuries are observed in plants suffering from dehydrative stress, such as concentration of solutes within cells, changes in cell volume and membrane shape, disruption of water potential gradients, inhibition of photosynthesis, loss of turgor, disruption of membrane integrity, denaturation of proteins and oxidative stress (Bray, 1997; Dat *et al.*, 2000).

Active oxygen species

Overproduction of active oxygen species (AOS) is a general phenomenon in plants suffering various types of stress, including drought, salinity and low temperatures. The accumulation of AOS under these conditions results mainly from a decline in CO_2 fixation, leading to higher leakage of electrons to O_2. Reduction of O_2 proceeds through several steps and forms superoxide (O^{*-}_2), hydrogen peroxide (H_2O_2) and the hydroxyl radical (OH^-). The hydroxyl radical is the strongest oxidizing agent known and is formed from hydrogen peroxide in the presence of a metal catalyst, most commonly Fe^{2+} through Fenton or Haber-Weiss-like reactions (Fenton, 1894; Fenton, 1899; Haber and Weiss, 1934). The AOS cause damage to DNA, proteins and lipids (Richter and Schweizer, 1997). Plants have several protective mechanisms against AOS; antioxidant- enzymes and metabolites. Superoxide dismutase catalyzes the dismutation of superoxide to hydrogen peroxide and water while catalase catalyzes the removal of hydrogen peroxide into water and oxygen (Scandalios *et al.*, 1997; Van Camp *et al.*, 1994). Another system, the ascorbate-glutathione cycle is involved in the removal of hydrogen peroxide (Noctor and Foyer, 1998).

The protective system can be used for improving stress tolerance, for example transgenic alfalfa overproducing a Mn-superoxide dismutase showed reduced injury from water deficit and freezing stress (McKersie *et al.*, 1996).

Membranes

Membrane damage can be caused by chilling and dehydrative stresses such as freezing, drought and salinity. Chilling stress causes membranes to undergo a phase transition from the liquid crystalline to the solid crystalline state (Levitt, 1980). This transition restricts the movement of small molecules through the membrane and therefore decreases the permeability to water and aqueous solutes. Phase transition causes several metabolic dysfunctions that are secondary in nature. Chilling resistance is due to an ability to maintain the membrane lipids in the liquid crystalline state at chilling temperatures. This is commonly brought about by an increase in the unsaturated fatty acid content of the membrane (Levitt, 1980). The importance of unsaturated fatty acids is evident in the

Arabidopsis mutants *fad2*, *fad5* and *fad6* which have reduced amounts of unsaturated fatty acids and are more susceptible to chilling (Hughly and Somerville, 1992; Miguel *et al.*, 1993).

By altering the fatty acid composition it is possible to increase the chilling tolerance, for example tobacco overproducing a bacterial Δ9 desaturase had reduced levels of saturated fatty acids and an increased chilling tolerance (IshizakiNishizawa *et al.*, 1996).

Freeze induced dehydration and drought leads to more severe membrane damage. Efflux of water from cells results in shrinkage of cell walls and plasma membranes and eventually to collapse of cells. Osmotic stress (salt) acts through a different mechanism, the cell wall remains intact whereas the plasma membrane shrinks as a result of water loss from within cells (Palta and Weiss, 1993).

Osmolytes

Osmolytes are normally small organic molecules, highly soluble and non toxic to cells in high concentrations. Osmolytes are also called, osmo-protectants, compatible solutes or simply solutes. Metabolites that serve as osmolytes include polyhydroxylated sugar alcohols, amino acids and their derivatives, tertiary sulphonium compounds and quaternary ammonium compounds (Bohnert and Jensen, 1996). Accumulation of osmolytes lowers the water potential, and cells can maintain their water content under mild dehydration, thus maintaining turgor. Osmolytes are also believed to protect lipids and proteins under low water content by acting as a replacement for water. In this way, the hydrophobic-hydrophilic orientation of the membrane phospholipids can be maintained and protein aggregation prevented (Bohnert and Jensen, 1996; Crowe and Crowe, 1984). Extreme desiccation promotes crystallization of proteins and solutes and one way to avoid this is vitrification (glass formation). Osmolytes (sugars) are believed to promote vitrification as water is removed from cells. A glass phase does not have a thermal transition to a solid phase at low temperatures and is a liquid solution with the viscosity of a solid. The glass phase prevents collapse of cells, maintains macromolecular structures and traps molecules (McKersie and Leshem, 1994).

Considerable interest has been focused on the protective effect of osmolytes and enzymes that synthesize different osmolytes have been introduced into plants in order to improve tolerance. For example, transgenic tobacco transformed with a gene encoding mannitol 1-phosphate dehydrogenase accumulated mannitol and had an increased ability to tolerate high salinity. Tobacco overproducing trehalose-6-phosphate synthase accumulated trehalose, and an increased drought tolerance was observed (Holmstrom *et al.*, 1996).

Abscisic acid

The concentration of abscisic acid increases in plants subjected to water deficit stress (Lang *et al.*, 1994; Zeevart and Creelman, 1988; Ryu *et al.*, 1995). The initial response to water deficit is stomatal closure to stop transpiration to maintain water balance. Stomatal closure is a rapid and direct ABA regulated

event compared to slow events that require RNA and protein synthesis (Zeevart and Creelman, 1988). The importance of stomatal closure is evident in the Arabidopsis mutants; abscisic acid deficient 1 (*aba1*) and abscisic acid insensitive 1 (*abi1*) (Koorneef *et al.*, 1982; Koorneef *et al.*, 1984). These mutants exhibit a drought sensitive phenotype caused by enhanced transpiration due to failure in stomatal closure (Koorneef *et al.*, 1982; Koorneef *et al.*, 1984).

The endogenous level of ABA increases transiently during the cold acclimation process in Arabidopsis (Lang *et al.*, 1994; Zeevart and Creelman, 1988). It is evident that there exist a cross talk between low temperature and water stress since both drought and salt stress can increase freezing tolerance of Arabidopsis and potato respectively (Mantyla *et al.*, 1995; Ryu *et al.*, 1995). It has been shown that the *aba1* mutant is unable to cold acclimate and that the *abi1* mutant has impaired ability to cold acclimate (Heino *et al.*, 1990; Mantyla *et al.*, 1995). The freezing tolerance of the *aba1* mutant and wild type can be increased by application of exogenous ABA indicating a role of ABA in cold acclimation (Mantyla *et al.*, 1995). However the fact that ABA treatment mimics cold acclimation does not necessarily mean that ABA plays a role in low temperature adaptation (Mantyla *et al.*, 1995; Thomashow, 1999). Impairment of freezing tolerance in the *aba1* and *abi1* mutants can be due to a pleiotropic effect since the mutants display a wilted phenotype and reduced vigor (Gilmour and Thomashow, 1991; Mantyla *et al.*, 1995; Thomashow, 1999).

In addition of being responsive to abiotic stress ABA play an important role during seed maturation (Koorneef and Karssen, 1994)

Protein accumulation

Adaptation to environmental change is crucial for plant growth and survival. The exact molecular and biochemical mechanism of adaptation is poorly understood. The most notable response observed in plants exposed to water deficit is the high accumulation of novel proteins. Many stress-induced genes have been cloned and found to encode enzymes required for biosynthesis of osmoprotectants, LEA proteins, antifreeze proteins, chaperones, detoxification enzymes, transcription factors, kinases and enzymes involved in phosphoinositide metabolism. (Bray, 1997; Hasegawa *et al.*, 2000; Ingram and Bartels, 1996; Thomashow, 1998 and references therein).

Dehydrins

The first two *DHN* genes cloned and characterized were *RAB21* (responsive to ABA) from rice and *D-11* from cotton (Baker *et al.*, 1988; Mundy and Chua, 1988). The cotton D-11 is classified as a late embryogenesis abundant (LEA) protein and the term LEA D-11 is sometimes used for DHNs (Baker *et al.*, 1988). Dure *et al.* (1989) classified LEA proteins into three groups and the DHNs were classified as LEA group 2, often referred to as LEA (II). Consequently, four names are in use for this protein family, RAB, LEA D-11, LEA (II) and DHNs.

The term DHN for these proteins was first suggested in 1989 and has since found widespread use (Close *et al.*, 1989). Although the term DHN was initially intended for dehydration-induced proteins, today proteins are classified as DHNs based on sequence homology rather than after their expression characteristics. In addition to being produced during the later stages of embryogenesis and in mature seeds. DHNs have been found to accumulate in plants treated with abscisic acid, salt, drought and low temperature.

Genes encoding DHNs have been cloned from both angiosperms and gymnosperms, and there is genetic evidence of DHNs from lower land plants such as the moss *Tortula ruralis* (AF275946) and immunological evidence of dehydrins in cyanobacteria (Close and Lammers, 1993), algae (Li *et al.*, 1997), ferns (Reynolds and Bewley, 1993) and liverworts (Hellwege *et al.*, 1994).

DHNs show no sequence similarity to any known protein found in the databases and appear to be confined to photosynthesizing organisms. The protein family database has a collection of 147 DHNs and a search of the EMBL database in December 2000 using the search term “dehydrin” yielded 197 hits. Considering the fact that there exist multiples of some genes and both cDNA and genomic sequences, the actual number is probably slightly lower. However, several DHN genes are not indexed as DHNs in the database. Overall, a large effort has been made to clone and characterize DHNs from numerous plant species.

DHNs appear to be a redundant protein family. In barley for instance 12 DHN genes have been cloned and characterized (Choi and Close, 2000; Choi *et al.*, 1999). In Arabidopsis six *DHN* genes have been cloned and characterized *LTI29/ERD10* (Kiyosue *et al.*, 1994; Welin *et al.*, 1994), *LTI30/XERO2* (Rouse *et al.*, 1996; Welin *et al.*, 1994), *XERO1* (Rouse *et al.*, 1992) *COR47* (Gilmour *et al.*, 1992; Welin *et al.*, 1995), *ERD14* (Kiyosue *et al.*, 1994) *RAB18* (Lang and Palva, 1992). In addition there is an EST clone *PAP310* (X91920) and in the Arabidopsis database we found two new potential DHNs not previously characterized.

Structure

Dehydrins are structurally characterized by three conserved sequences; the Y-, S- and K-segments, reviewed in (Close, 1996; Close, 1997). In the most N-terminal part, the Y-segment (V/TDEYGNP, sometimes referred to as DEYGNP), can be found in one to three copies. Downstream of the Y-repeat, five to seven serine residues followed by three acidic amino acids form the S-segment. The K-segment (EKKGIMDKIKEKLPG) is the only segment present in all dehydrins. The K-segment has been proposed to form an amphiphatic α-helix by helical-wheel analysis (Dure, 1993). Furthermore, by reducing the 15 amino acid consensus shown above to 10 (IMDKIKEKLP) or 12 (GIMDKIKEKLPG) residues, the K-segment was proposed to form a class A amphiphatic α-helix (Close, 1996). Class A amphiphatic helices have well demarcated polar and nonpolar faces with negatively charged residues opposite the hydrophobic face and positively charged residues at the polar non polar interface (Segrest *et al.*,

1990). The amino acids separating the conserved segments are termed ϕ-segments. These segments are not preserved segments and show considerable variation between different DHNs. There are five distinct structural types of DHNs using the YSK shorthand, Y_nSK_2, K_n, SK_n, K_nS and Y_2K_n (Close, 1996). Some other notable characteristics of DHNs are the lack of the amino acids cysteine and tryptophan, high percentage of charged and polar amino acids (hydrophilic) and the ability to remain in solution after boiling (Close, 1996; Close, 1997; Close *et al.*, 1989).

Dehydrins have been purified from plants (Table 1) and by expression of recombinant DHNs in *Escherichia coli* (Table 2). Structural analyses have been done for three DHNs and all demonstrate an apparent lack of a defined structure under the conditions tested. Circular dichroism analysis has shown that maize DHN G50 (probably RAB17) contained about 75% disorder consistent with a protein that to an extraordinary degree lacks ordered structure (Ceccardi *et al.*, 1994). Ismail *et al*. (1999) analyzed a 35 kDa cowpea DHN and it lacked a defined structure. However, in the presence of SDS an α-helical structure was detected. Additionally, ^{1}H-NMR spectra demonstrated that DSP16 from *C. plantagineum* is essentially unstructured (Lisse *et al.*, 1996). Choi *et al.* (1999) compared the allelic differences in barley DHNs and noted that most changes occurred mainly as duplications or deletions of the ϕ-sequences separating the conserved repeats. The authors suggested that the Y-, S-, K-, and ϕ-segments represent structures (domains) with considerable constraint and therefore DHNs in their native state do have a specific structure (Choi *et al.*, 1999). Oligomeric behavior has been demonstrated for the maize DHN G50 (17.7 kDa), which eluted with an apparent MW of 42 kDa using gel filtration chromatography (Ceccardi *et al.*, 1994) and spinach COR85 (probably CAP85) which eluted as a 350 kDa homo-tetramer (Kazuoka and Oeda, 1994). In contrast DSP16 was found to behave as a monomer in dynamic light scattering and sedimentation analysis (Lisse *et al.*, 1996).

Post-translational modifications

Phosphorylation of a DHN was first reported for maize RAB17 (Vilardell *et al.*, 1990). Plana *et al.* (1991) later demonstrated by *in vitro* and *in vivo* phosphorylation of RAB17 and subsequent peptide mapping that the phosphorylated polypeptide fragment contained the S-segment. The three acidic amino acids following the serine residues were initially identified as a putative casein kinase2 substrate sequence further supporting these results. Mutations in the consensus sequence also showed no or extremely low incorporation of phosphorus (Jensen *et al.*, 1998). RAB17 also possess a basic sequence stretch (RRKK) resembling a nuclear localization signal binding domain found in mammalian nucleolar phosphoprotein Nopp140 and yeast NSR1 (Goday *et al.*, 1994). Binding of RAB17, to peptides containing the nuclear localization signal of the simian virus 40 antigen was shown to be dependent on phosphorylation of RAB17 (Goday *et al.*, 1994). In-frame fusions of RAB17 to the reporter gene GUS directed GUS to the nucleus and cytoplasm in transgenic Arabidopsis.

Constructs mutated in the casein kinase2 recognition sequence demonstrated that phosphorylation of the S-segment is the key for nuclear location (Jensen *et al.*, 1998). Two other DHNs have also been shown to be phosphorylated, the tomato TAS14 (Godoy *et al.*, 1994) and DSP16 from *C. plantagineum* (Lisse *et al.*, 1996).

Interestingly, DHN-like proteins from blueberry and pistacio have recently been found to be glycosylated, a post-translational modification not previously reported (Golan-Goldhirsh, 1998; Golan-Goldhirsh *et al.*, 1998; Levi *et al.*, 1999). One of the blueberry DHNs has been cloned and the gene encodes a K_5 DHN (Levi *et al.*, 1999).

Localization of dehydrins

Tissue distribution of dehydrins

The tissue distribution of DHNs has been analyzed in several plant species by immuno-histochemical methods. Dehydrin P-80 in cold acclimated barley was found to be localized in vascular tissue and epidermis of shoots and in non acclimated leaves in the vascular bundle (Bravo *et al.*, 1999). Schneider *et al*. (1993) analyzed the localization of DSP16 and DSP16-like DHNs in drought treated *C. plantagineum.* Staining was detected in all types of cells but preferentially in cytoplasm-rich cells like phloem sieve tube elements in leaves, and embryonic cells in the seed. ECP40, a carrot DHN, was localized in the endosperm and zygotic embryos in mature seeds (Kiyosue *et al.*, 1993). Proteins of the WCS120 DHN family were localized mainly in the vascular bundle and bordering parenchymal cells in cold acclimated wheat crown tissues (Houde *et al.*, 1995). Another wheat DHN (WCOR410) was also found to accumulate preferentially in the vascular transition area (Danyluk *et al.*, 1998). Peach PCA60 appeared to be generally distributed in cells of all tissues of shoots collected in January, including epidermal, cortical, phloem and xylem tissues (Wisniewski *et al.*, 1999). Immuno-localization in salt-stressed tomato plants showed strong accumulation of TAS14 in developing adventious root primordia, in vascular tissue of the shoot and in differentiated cortical cells of the stems and leaves (Godoy *et al.*, 1994). The maize dehydrin RAB17 was localized in all cell types of mature embryos (Goday *et al.*, 1994). The conclusion from all these studies is that most of the DHNs are localized in vascular tissue.

Subcellular localization of dehydrins

To gain a better understanding of the role played by DHNs in plants, the subcellular localization has been thoroughly analyzed. Several studies demonstrate that DHNs are only present in the cytosol and the nucleus. Examples of such studies are maize embryos (Asghar *et al.*, 1994; EgertonWarburton *et al.*, 1997; Goday *et al.*, 1994), developing tomato root primordia (Godoy *et al.*, 1994), pea root tip meristems subjected to slow dehydration (Bracale *et al.*,

1997), wheat crown tissues (Houde *et al.*, 1995) and peach shoots (Wisniewski *et al.*, 1999). The nuclear localization of maize RAB17 is dependent on phosphorylation of the S-segment (Jensen *et al.*, 1998). Interestingly, most DHNs containing the S-segment possess similar NLS sequences as found in RAB17 and it is likely that nuclear targeting is similar to the one demonstrated for RAB17 in many of these DHNs. There are however some DHNs that have been found only in the cytosol such as DSP16 in desiccated leaves of the resurrection plant *C. plantagineum* (Schneider *et al.*, 1993) and rice RAB21 (fractionation study) (Mundy and Chua, 1988).

The wheat DHN WCOR410 was found to accumulate in the vicinity of the plasma membrane of cells in the vascular transition area (Danyluk *et al.*, 1998). Recent studies also demonstrate the presence of DHNs in or associated with organelles; the peach PCA60 was, in addition to the cytosol and nucleus, also found to be associated with chloroplasts (Wisniewski *et al.*, 1999) and fractionation studies of winter wheat, winter rye and maize showed the presence of two DHN-like proteins associated with mitochondria (Borovskii *et al.*, 2000). Another fractionation study showed the presence of spinach CAP85 predominately in the cytosol but also associated to the endoplasmic reticulum (Neven *et al.*, 1993). In non-acclimated birch a 16 kDa constitutive DHN was localized in the cytoplasm whereas after cold acclimation two additional DHNs were detected and the three DHNs were localized in the nuclei, storage protein bodies and starch-rich amyloplasts (Rinne *et al.*, 1999). In maize one study has demonstrated DHNs associated to protein and lipid bodies in addition to the cytosol and nucleus (EgertonWarburton *et al.*, 1997). In summary DHNs appear to be found in or associated to most cellular organelles and their localization can be affected by post translational modifications such as phosphorylation

Dehydrins: functional studies and proposed function

Functional studies of dehydrins

The contribution of DHNs to abiotic stress tolerance has been tested in transgenic plants and in yeast by overproducing single DHNs. Tobacco overproducing spinach CAP85 was evaluated for relative freezing tolerance by measurement of the electrolyte leakage on detached leaves subjected to controlled freezing, the authors conclude that CAP85 had no profound influence on stress tolerance (Kaye *et al.*, 1998). Similar results were obtained when overproducing RAB18 and LTI29 separately in both Arabidopsis and tobacco (Lång, 1993; Welin, 1994). Drought tolerance of tobacco overproducing *C. plantagineum* DSP16 was evaluated by mild drought stress on detached leaves following determination of electrolyte leakage, no increase in drought tolerance was detected (Iturriaga *et al.*, 1992). The authors suggest that this could be due to an unsuitable test system (ion-leakage) or a requirement for simultaneous expression of several drought-related proteins (Iturriaga *et al.*, 1992). No improvement in drought tolerance was detected by overproducing LTI29 in Arabidopsis using a similar test system

(Lång, 1993; Welin, 1994). The drought tolerance of tobacco and Arabidopsis overproducing RAB18 was estimated visually on detached leaves and no difference was detected compared to wild type plants (Lång, 1993). Constitutive expression of a barley DHN (*ABA2*) in Arabidopsis enhanced the germination rate under salt and osmotic stress but no significant difference in germination at low temperature or in freezing damage of adult plants was detected (Calestani *et al.*, 1998). Expression of the tomato *LE4* in yeast conferred tolerance to high concentrations of KCl but not to NaCl or sorbitol. In addition, the yeast strain producing LE4 had increased freezing tolerance (Zhang *et al.*, 2000). Taken together, ectopic expression of DHNs in plants have so far not improved freezing or drought tolerance, the only improvement obtained is an increased germination under salt and osmotic stress.

Biochemical analyses of DHNs have shown that spinach COR85, maize G50, wheat WSC120 and peach PCA60 have cryoprotective activity (Close, 1996; Houde *et al.*, 1995; Kazuoka and Oeda, 1994; Wisniewski *et al.*, 1999). The cryoprotective activity was measured in a lactate dehydrogenase freeze-thaw test (Carpenter and Crowe, 1988). In addition to cryoprotective activity, PCA60 was demonstrated to posses antifreeze activity by modifying the normal growth of ice and exhibiting thermal hysterisis (Wisniewski *et al.*, 1999).

Proposed function of dehydrins

The role of DHNs in stress tolerance remains to be established. Overproduction of DHNs in tobacco and Arabidopsis did not contribute to an increase in drought or freezing tolerance, see previous section. Based on the accumulation data DHNs are believed to play a role in plants subjected to drought, salt and low temperature stress, and in the desiccated seed. Theories of DHN function have been presented and the main view is that DHNs stabilize membranes and proteins during dehydrative conditions thereby protecting the integrity of the cell.

In one of the first papers describing the cloning of a *DHN* gene (cotton *D-11)*, Baker *et al.* (1988) conclude that LEA proteins probably does not have an enzymatic function due to the biased amino acid composition. Furthermore they suggest that some of the LEA proteins might function to mitigate the physiochemical problems encountered in the plant cytosol during dehydration by protecting the structural integrity of membranes and proteins. The LEA proteins D-11 and D-113 are also suggested to act by solvating cytosolic structures, this idea is based on the high amount of glycine and serine residues and it is suggested that both proteins may exist as random coils and through their hydroxyl group solvates structural surfaces (Baker *et al.*, 1988).

The K-segment was first proposed to form an amphiphatic α-helix and later suggested to form a class A amphiphatic α-helix (Close, 1996; Dure, 1993), see previous section; *structure* for details. Class A amphiphatic helices are found in apolipoproteins and is responsible for interactions to lipids. This lead Close (1996) to suggest that one possible role of the K-segment is interaction with membranes and partially denatured proteins, that DHNs are solubilizing agents with detergent and chaperone properties. Close and colleagues purified a 35 kDa

cowpea DHN and showed that an α-helical structure was induced in the presence of SDS further supporting their suggestion (Ismail *et al.*, 1999). Cowpea lines with allelic variation of the 35 kDa DHN demonstrated that presence of the 35 kDa DHN was associated to chilling tolerance during seedling emergence (Ismail *et al.*, 1999). The increased chilling tolerance observed was not correlated to reduced electrolyte leakage indicating that the effect might not be protection of the plasma membrane. Instead it is suggested that the DHN interacts with membranes in the interior of the cells and reduces dehydration induced damages (Ismail *et al.*, 1999). Furthermore, it is suggested that DHNs may act in a similar manner as α-synuclein (Ismail *et al.*, 1999). Alpha-synuclein is a highly conserved presynaptic protein that have random conformation in solution but upon binding to lipids an increase in the α-helicity can be observed (Davidson *et al.*, 1998).

Wheat DHN WCOR410 was found to associate to the plasma membrane and is suggested to stabilize plasma membranes during freezing (Danyluk *et al.*, 1998). The exact mechanism of how WCOR410 protects the plasma membrane is not known, three possible modes was suggested; (i) by replacing water and thereby solvate membranes, (ii) prevent interactions between membrane bilayers, reducing fusion of membranes and lamellar to hexagonal II phase transition or (iii) by forming salt bridges to ions preventing the damaging effect by increased ionic concentrations (Danyluk *et al.*, 1998).

Taken together DHNs are believed to stabilize membranes and proteins during dehydrative conditions thereby protecting the integrity of the cell.

Table 1. Purification of native dehydrins.

Species	Tissue	DHN	Purification scheme	Ref.
Peach	bark	PCA60	prIEF, prSDS	(1)
Cowpea	seeds	35 kDa	HF, CIEC, HIC, AIEC	(2)
Maize	kernels	G50	HF, CIEC, HIC, GF	(3)
	embryos	RAB17	ASF, CIEC, ASF, AIEC	(4)
Blueberry	buds	65, 60 kDa	prIEF, prSDS	(5)
Pistacio	buds[1]	IBP 32 kDa	AcP, GF	(6)
		IBP 27 kDa	AcP, GF	(6)
Spinach	hyp/cot	CAP85	prIEF, AcP	(7)
	leaves	COR85	HF, ASF, AIEC, CIEC	(8)
Soybean	seeds	AS26k	ASF, CIEC, GF, HIC	(9)

Footnote: [1]protein extracts were defatted prior to purification. Abbreviations used; AcP. Aceton precipitation, AIEC, anion exchange chromatography; ASF, ammonium sulphate fractionation; CIEC, cation exchange chromatography; HF, heat fractionation; HIC, hydrophobic interaction chromatography; prIEF, preparative isoelectric focusing, prSDS, preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis.

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Table 2. Purification of recombinant dehydrins

Species	DHN	tag	Purification steps	Ref.
Maize	DHN1	no	HF, CIEC, HIC	(1)
Tomato	TAS14	C-LZ	IB	(2)
	TAS14	GST	AC[1]	(2)
Wheat	WCOR410	his	IMAC, prSDS	(3)
	WCS120	no	HF, prSDS	(4)
C. plantagineum	DSP16	no	AIEC, CIEC, RPC	(5)
	DSP16	GST	IB, prSDS	(6)
Potato	CI7	his	IMAC[2]	(7)
Rice	RAB21	β-gal	AC[3]	(8)
Arabidopsis	RAB18	MBP[4]	HF, AC[5], prSDS	(9)
	ERD14	his	HF, IMAC, prSDS	(II)
	LTI29	his	HF, IMAC, prSDS	(II)
	LTI30	his	HF, IMAC, prSDS	(II)
	COR47	his	HF, IMAC, prSDS	(II)
	RAB18	no	HF, IMAC, AIEC	(I)
	LTI29	no	HF, IMAC, AIEC	(I)
	LTI30	no	HF, IMAC, CIEC	(I)
	COR47	no	HF, IMAC, AIEC	(I)

Footnotes: [1]glutathione resin, [2]purified under denaturing conditions, [3]anti-β-galactosidase resin, [4]exported to the periplasmic space, [5]amylose resin. Abreviations used; AC affinity chromatography, AIEC anion exchange chromatography; β-gal β-galactosidase, CIEC cation exchange chromatography, C-Lz Cro-LacZ, GST glutathione S-transferase, HF heat fractionation, HIC hydrophobic interaction chromatography, IB inclusion bodies, IMAC immobilized metal ion affinity chromatography, MBP maltose binding protein, prSDS preparative sodium dodecyl sulphate polyacrylamide gel electrophoresis, RPC reverse phase chromatography.

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Aims of the present study

DHNs are believed to play a fundamental role in the plant response and adaptation to abiotic stress that leads to cellular dehydration, such as drought, low temperature and salinity. Consequently almost 200 *DHN* genes have been cloned and characterized. Despite this body of information, the actual physiological function of DHNs remains elusive. The main goal of my studies has been to investigate DHNs in order to obtain information which would help us in the elucidation of their actual biological function. To achieve this goal I have studied DHNs using several different approaches listed below. In the following results and discussion section I will go through my results step by step and try to discuss them in the context of results obtained in other laboratories and their contribution to our overall understanding.

Aims:

- Produce protein-specific antibodies against several members of this protein family in Arabidopsis, in order to get a clearer picture of accumulation and tissue-localization in one plant species.

- Perform biochemical analysis on purified recombinant proteins.

- Generate transgenic Arabidopsis overproducing more than one DHN to examine the possible contribution of DHNs in abiotic stress tolerance.

- Clone *DHN* genes from *P. patens*. Firstly to see if DHNs exist in lower land plants and secondly to generate a gene-specific mutant by homologous recombination.

Results and discussion

Protein function can be studied using both genetic and biochemical approaches. The most powerful method is to create mutants that can be used for evaluation of phenotypic differences and thus give an insight to the physiological role/function of a specific protein. The recent wealth of information regarding plant protein function is mostly based on mutant studies in Arabidopsis where a clear-cut phenotype has been reported. Mutants in Arabidopsis have been made by chemical mutagenesis, radiation, T-DNA tagging and transposon mutagenesis. Another genetic approach to study protein function is the use of transgenic plants where the gene of interest is overexpressed or silenced. If the protein under investigation has been characterized in another organism then database searches can be a useful tool. Complementation analysis in another organism such as yeast can for example give valuable data regarding protein function. A third method,

until recently not available in plants, is the creation of mutants by the generation of gene-specific mutants by homologous recombination. This technique is now available following the discovery that the moss *P. patens* can undergo homologous recombination at high frequency (Schaefer *et al.*, 1991). The use of gene targeting in *P. patens* will undoubtedly make it far easier to obtain specific mutants and will help to elucidate the function of many proteins in plants.

The genetic approach is unquestionably very useful for studying protein function nevertheless purified proteins are a necessity for detailed biochemical studies. Purified proteins are not only important for biochemical analysis but can also be used for the production of antibodies. A specific antibody against a protein of interest is a useful complementary tool for studies of protein function where localization based on immuno-cytochemistry can give valuable clues regarding the actual function. The last requirement, but perhaps the most difficult part of elucidating a function for a protein, is a good hypothesis that can be experimentally tested and either verified or discarded.

Purification of his-tagged dehydrins (II)

As mentioned previously, our first strategy to gain information on the possible physiological function of DHNs involved the localization studies of several members of this protein family in Arabidopsis. The first step in such a study involves the purification of the proteins to generate antibodies. To simplify purification of DHNs, the corresponding full-length cDNAs of *LTI29* (Welin *et al.*, 1994), *LTI30* (Welin *et al.*, 1994), *COR47* (Gilmour *et al.*, 1992; Welin *et al.*, 1995) and *ERD14* (Kiyosue *et al.*, 1994) were cloned to a vector encoding an N-terminal His-tag. Expression of His-tagged proteins was performed in *E. coli* under the control of an IPTG-inducible promoter. His-tagged proteins were found to be soluble without any sign of inclusion bodies, in agreement with most previous studies (Danyluk *et al.*, 1998; Houde *et al.*, 1992; Jepson and Close, 1995; Kirch *et al.*, 1997; Lisse *et al.*, 1996). Two exceptions have however been reported; TAS14 which was purified as a Cro-LacZ fusion using the pEX1 expression system, a system designed for production of inclusion bodies (Godoy *et al.*, 1994) and purification of DSP16 fused to glutathione-S-transferase (GST) (Schneider *et al.*, 1993).

Close *et al.* (1989) demonstrated that DHNs remain soluble upon boiling, a property that can be used as an initial step in the purification of DHNs. Heat fractionation was therefore used as an initial purification step followed by immobilized metal ion affinity chromatography (IMAC) using a Cu-IDA-Sepharose column (hereafter referred to as Cu-column). Other reports of purification of His-tagged DHNs with the aid of IMAC have been presented. Danyluk *et al.* (1998) purified WCOR410, a SK_3 DHN as a His-tagged fusion protein and Lisse *et al.* (1996) reported that purification of His-tagged DSP16 (YSK_2) failed because of low yield and proteolytic degradation. His-tagged CI7

(SK_3), a potato DHN, was purified under denaturing (UREA) conditions (Kirch *et al.*, 1997).

Surprisingly, the His-tagged DHNs bound very strongly to the Cu-column. The only way to elute the recombinant proteins was by stripping the Cu-column with EDTA, thereby eluting the Cu(II) ions together with the recombinant proteins. The Cu-EDTA fractions were thereafter separated on preparative SDS-PAGE gels and the corresponding protein bands were excised and used for immunization of rabbits to generate polyclonal antibodies against ERD14, LTI29, LTI30 and COR47.

Purification of recombinant dehydrins (I)

The strong binding of DHNs to the Cu-IDA-Sepharose column caught our interest and we decided to study this phenomenon more thoroughly. His-tagged constructs were unsuitable for protein-metal binding studies so we made new gene constructs to express *RAB18*, *LTI29*, *LTI30* and *COR47* (*RAB18R*, *LTI29R*, *LTI30R* and *COR47R*) in *E. coli* lacking the His-tag. In this study we included RAB18 (Lang and Palva, 1992) since none of the proteins LTI29, LTI30 or COR47 harbors the Y-segment and we wanted to study all three types of DHNs found in Arabidopsis, represented by LTI30 (K-type), LTI29 and COR47 (SK-type) as well as RAB18 (YSK-type). The recombinant proteins did not harbor any additional or changed amino acids compared to the native proteins except for LTI30 where a single glycine residue was added after the initial methionine for cloning purposes.

Expression of the recombinant proteins in *E. coli* resulted in accumulation of soluble proteins. As a first purification step, bacterial protein extracts were heat-fractionated at a lower temperature (55°C) than the 85°C used by Jepson and colleagues (Jepson and Close, 1995) to minimize the risk of chemical modifications such as deamidation, hydrolysis and oxidation (Marshak *et al.*, 1996). Initially we tried to purify the recombinant DHNs without the use of IMAC, avoiding a potential problem of removal of Me(II) ions prior to analysis. Purification was done using ion exchange chromatography followed by gel filtration. However the purity level reached only 90-95 %, and this was not deemed as sufficient for further *in vitro* analysis. DHNs have a low absorbancy at 280 nm because they lack tryptophan residues, 10% impurities would therefore give inaccurate values in concentration measurements using absorbancy at 280 nm. Due to the low purity obtained with ion exchange and gel filtration chromatography, we decided to purify the recombinant proteins on a Cu-column. Recombinant proteins, lacking the His-tag were found to bind firmly to the column and could only be eluted with EDTA.

The two amino acids generally involved in metal binding in proteins are cystein and histidine. Since DHNs lack cysteines, the observed binding is probably due to the number and the surface accessibility of histidine residues in these proteins. The metal binding capacity of histidine residues is demonstrated

in certain species of *Alyssum* (*Brassicae*) where free histidines have been reported to chelate Ni(II) (Krämer *et al.*, 1996). More evidence of the high capacity of histidines in binding metal ions is shown by the development of IMAC, where addition of histidine residues at the N-terminal or C-terminal end of almost any protein can be used to purify that protein (Hochuli *et al.*, 1987). Analysis of the primary sequence of the four DHNs reveals that LTI30 contains 26 histidines of which 22 form His-His pairs. Tandem histidines have previously been reported to bind metals more strongly than single His-residues (Porath, 1992). The other DHNs in this study also have the potential to bind metals via their histidine residues since, RAB18 has 8 histidines (3 pairs), LTI29 contains 11 histidines (0 pairs) and COR47 harbors 13 histidines (2 pairs). Furthermore, the strength of the binding could be linked to the number and surface accessibility of these residues. More results from the IMAC tests are presented in the next section, *analysis of purified proteins.*

To remove the Cu(II) ions, the Cu-EDTA fractions were precipitated with ammonium sulphate followed by a buffer exchange (group separation) step on a Sephadex G-25 column. This step allowed subsequent application and separation on ion exchange resins. LTI30R with a pI of 9.4 was purified by cation exchange chromatography whereas RAB18R (pI of 7.9), LTI29R (pI of 4.9) and COR47R (pI of 4.8) were purified by anion exchange chromatography. Both LTI29R and COR47R eluted as broad peaks, in fact LTI29R eluted as two close peaks. This phenomenon could be due to aggregation. Purification of a cowpea DHN also resulted in a broad peak using ion exchange chromatography (Ismail *et al.*, 1999). Previously, only two DHNs have been purified on a large scale from bacteria, maize DHN1/RAB17 and *C. plantagineum* DSP16, and the yields per liter of bacterial culture were 1.2 and 5 mg respectively. DHN1/RAB17 and DSP16 belong to the YSK type of DHNs as does RAB18R in our study. The yield of RAB18R was 2.8 mg per liter, comparable with the results of DHN1/RAB17 and DSP16. No study has previously described large scale purification of a K or SK type of DHN without the use of an affinity tag. The yields per liter of bacterial culture (4.2 mg for LTI30R (K type) and 6.3 and 12.5 mg for LTI29R and COR47R, respectively both representing SK types of DHNs) are sufficient for biochemical studies *in vitro*. The identity of the purified DHNs was verified by N-terminal sequencing and by mass spectrometry. Purity was controlled by reverse phase chromatography and found to be over 95%. In conclusion we had obtained four purified DHNs suitable for biochemical analysis.

Analysis of purified proteins (I and unpublished results)

Analytical gel filtration under native conditions indicated that the recombinant DHN proteins migrated as oligomers. Oligomeric behavior has been demonstrated for the maize DHN G50 (17.7 kDa), which eluted with an apparent MW of 42 kDa using gel filtration chromatography (Ceccardi *et al.*, 1994). In contrast DSP16 was found to behave as a monomer in dynamic light scattering

and sedimentation analysis (Lisse *et al.*, 1996). To determine whether the Arabidopsis proteins were true oligomers we performed gel filtration under denaturing conditions in order to dissociate potential oligomers. The elution volumes of the DHNs under denaturing conditions were lower than those observed under native conditions, showing that under denaturing conditions the Arabidopsis DHNs elute as larger proteins. If the DHNs tested were oligomers we would expect elution under denaturing conditions as the monomeric molecular weight. As this was not the case our results indicate that the DHNs tested are not oligomers and have an extended structure or perhaps a lack of structure (disorder).

In order to test the relative binding strength of the individual DHNs to different metals, we loaded equimolar amounts of the heat-fractionated extracts to an uncharged column and a column pre-charged with either Cu(II), Ni(II), Zn(II) or Co(II). Elution was done with a gradient of 1 - 10 mM EDTA. None of the DHNs were adsorbed onto the uncharged column. We found that binding to Cu(II) and Ni(II) columns were in the order LTI30R > RAB18R > LTI29R = COR47R. Binding to Co(II) and Zn(II) was only observed in the case of LTI30R. To further characterize the metal-binding property of the DHNs we wanted to determine the stoichiometry and the K_d of the proposed binding. Proteins and metals were mixed, followed by separation of unbound metals. The metal content of the protein solution was then determined by atomic absorption spectroscopy. Unfortunately we encountered problems due to aggregation of the DHNs after the addition of Me(II) ions. The only stoichiometry that could be determined was for aggregates, not for proteins in solution. To circumvent this problem we tried another approach using equilibrium dialysis since there was a possibility that the slow addition of Me(II) ions due to the dialysis would prevent aggregation. Equilibrium dialysis has the additional advantage that both the stoichiometry and the K_d can be determined in a single experiment. However, again no clear information was obtained and aggregates were formed as described previously. The aggregation was found to be reversible since addition of EDTA dissolved the aggregates.

It is possible that the DHNs form aggregates on the Me(II)-column and that the aggregates are dissolved by the addition of EDTA, similar to the *in vitro* binding experiments. We believe that the strong binding seen on Me(II)-columns could be a result of the flexible shape of DHNs and of the surface accessibility of the His-residues. Nevertheless, the use of IMAC is an excellent tool for purification of DHNs both recombinant and directly from plants. We have demonstrated that both LTI30 and COR47 from cold-acclimated Arabidopsis bind to a Cu(II) and Ni(II) column (data not shown). Further evidence supporting the use of IMAC in purification of DHNs comes from experiments with seed extracts of maize, winter wheat, spring wheat, barley and rye on a Cu-column. When analyzing EDTA-eluted fractions by western blot analysis we detected the same proteins in the EDTA-eluate as in seed extracts, thus demonstrating the power of IMAC as a tool for purification of DHNs from various plant species

Dehydrin protein and transcript accumulation (II)

As part of our strategy to clarify the possible function of DHNs in plants, we investigated whether the high transcript levels seen under stress conditions reflected the true DHN protein accumulation. The mRNA accumulation pattern of five Arabidopsis *DHNs* (*ERD14, RAB18, LTI29, LTI30* and *COR47)* were therefore determined in roots, stems and leaves of flowering plants subjected to salt, low temperature and ABA treatment. We found that *ERD14* was present in all samples, although mRNA levels were higher in stress-treated plants. This result is in contrast to a previous study describing *ERD14* accumulation upon ABA, drought and low temperature treatment but not during normal plant growth (Kiyosue *et al.*, 1994). *LTI30* transcripts were also detected in all samples, but transcript levels were much higher in low temperature-treated samples. In a previous study *LTI30* mRNA was found to accumulate mainly during low temperature stress, however in that study two-week old seedlings were analyzed which might explain the difference observed (Welin *et al.*, 1994). *COR47* and *LTI29* mRNA accumulated primarily in response to low temperature stress. Whereas RAB18 mRNA accumulated in plants treated with ABA or salt stress. In general, transcript levels were higher in roots and stems than in leaves.

To compare transcript levels with protein accumulation, plants of the same age as in the expression studies were subjected to ABA treatment, salt, and low temperature stress followed by de-acclimation at room temperature. In addition to roots, stems and leaves, flowers were also included in analyses of protein accumulation. The polyclonal antibodies against ERD14, LTI29, COR47 and LTI30 were all found to crossreact with the DHNs in Arabidopsis. In order to prevent this crossreaction, antibodies were affinity-purified for increased specificity to their specific protein. The COR47 antibodies could however, not be satisfactorily purified and were omitted from further studies. We were able to obtain a LTI29 antibody that was specific to LTI29 and the previously made RAB18 antibodies only recognized RAB18 (Mantyla *et al.*, 1995). Antibodies against LTI30 recognized, in addition to LTI30, RAB18 when this protein was present in high amounts. The ERD14 antibody was found to cross-react with COR47 to a certain extent. We could divide the DHNs tested into four classes based on the protein accumulation. Interestingly, those classes correlated with the conserved domains found in DHNs.

LTI30 (K_6) accumulates in response to low temperature stress and was found in all tissues tested. Accumulation was much higher in roots, flowers and stems than in leaves. The protein accumulation differed markedly from the accumulation of the *LTI30* transcript. Transcripts were detected in all samples (ABA, NaCl and low temperature-treated) including non-stressed plants, whereas the LTI30 protein was only detected after low temperature treatment. The difference observed between protein and mRNA levels indicates a post-transcriptional regulation of the *LTI30* gene. Post-transcriptional regulation has previously only been described for one *DHN*, *ci7* cloned in potato (Kirch *et al.*, 1997).

The RAB18 (Y_2SK_2) protein accumulated in response to ABA in all tissues tested and the highest protein level was detected in stems and leaves. In addition to the ABA accumulation, RAB18 was detected in salt-stressed flowers. RAB18 was the only DHN in this study detected in seeds. A previous study in our laboratory has shown that RAB18 accumulation in vegetative tissue is regulated by ABA since no or very reduced levels of protein are observed in the *aba1* and *abi1* mutants, respectively (Mantyla *et al.*, 1995). In this study we wanted to elucidate the role of ABA in RAB18 accumulation in seeds. We found that RAB18 accumulated in both the wild type and the ABA insensitive (*abi3-1)* mutant, but to a lesser extent in the ABA deficient (*aba1*) mutant. Another study has shown that *RAB18* expression in dry seeds is unaffected by the *abi5* mutant (Finkelstein and Lynch, 2000). This suggests that RAB18 accumulation in seeds is partially ABA dependent but independent of ABI3 and ABI5. Our results are in agreement with the expression analysis of the *RAB17* promoter fused to the GUS reporter gene in wild type Arabidopsis (LE) and *aba1, abi2* and *abi3* mutants (Vilardell *et al.*, 1994). GUS activity was detected in embryo and endosperm of both the wild type and the *abi* mutants but was lower in the *aba1* mutant (Vilardell *et al.*, 1994).

LTI29 and COR47, the SK_3 type of DHNs, accumulated primarily in response to low temperature. COR47 (detected with ERD14 antibodies) accumulated to about the same level in all tissues, whereas LTI29 showed a higher level of accumulation in flowers than in other tissues

ERD14 (SK_2) was detected in all samples, but protein levels were higher after exposure to salt, low temperature and ABA treatment. The highest level of ERD14 was detected in stems and leafs. Some other studies report the presence of constitutive DHNs, pea B61 an SK_2 DHN and DSP16 (YSK_2) from *C. plantagineum* (Robertson and Chandler, 1994; Schneider *et al.*, 1993). Constitutive DHN-like proteins have also been reported in birch (16 kDa), poplar (50 kDa) and dogwood (60 kDa) (Rinne *et al.*, 1999; Wisniewski *et al.*, 1996).

In summary, ERD14, RAB18, LTI29 and COR47 appear to be regulated at the transcriptional level whereas LTI30 seems to be both transcriptionally and post-transcriptionally regulated. However, further study, by for example nuclear run-on transcription, is required to clarify these observations.

Tissue and cellular localization of dehydrins (II)

To further characterize DHN accumulation in different tissues and cells of the plant, we performed immuno-histochemical localization of four DHNs in Arabidopsis (ERD14, RAB18, LTI30 and LTI29). Based on our previous western data we decided to characterize the localization of LTI29, LTI30 and ERD14 in unstressed and low temperature-treated plants, and of RAB18 in unstressed and ABA-treated plants. These localization studies showed tissue and cell type specific accumulations of DHNs in unstressed plants, which are summarized in table 3. Both ERD14 and LTI29 were found in the root tip and in the vascular

tissue of roots and stems. In addition, ERD14 was detected in the vascular tissue in leaves and flowers. RAB18 was found in the vascular tissue of stems and in stomatal guard cells in stems, leaves, and flower sepals and RAB18 appeared to be localized preferentially in the nuclei of guard cells. LTI30 was not detected in unstressed plants.

Table 3. Summary of the immunohistochemical localization studies of the LTI29, ERD14, RAB18 and LTI30 dehydrins in Arabidopsis.

DHN	Treatment	Root	Stem	Leaf	Flower
LTI29	unstressed	VT, RT	VT	ns	ns
ERD14		VT, RT	VT	VT	VT
RAB18		ns	VT, ST[1]	ST[1]	ST[1]
LTI30		ns	ns	ns	ns
LTI29	stressed	VT, GS, RT	VT, GS	VT, M	VT, GS,
ERD14		VT, GS, RT	VT, GS	VT, M	VT
RAB18		VT, GS, RT	VT, GS, ST[2]	VT, M, ST[2]	VT, GS, ST[2]
LTI30		VT, GS	VT	VT	VT, PS

Footnotes: [1]staining found in the nuclei, [2]staining found in both cytosol and nuclei.
Abbreviations used: DHN: dehydrin, GS: general staining, M: mesophyll cells, ns: no staining, PS: pollen sacks, RT: root tip, ST: stomatal guard cells, VT: vascular tissue.

Stress treatment resulted in a more general distribution of DHNs, although vascular tissue showed heavier staining (Table 3). LTI29 and ERD14 were detected in most cells of roots, stems and leaves. In flowers, ERD14 was only detected in the vascular tissue whereas LTI29 was found in most cells, including the vascular tissue. RAB18 was detected in most cells and in the vascular tissue in roots, stems, leaves and flowers. In addition RAB18 was detected in the root tip and in stomatal guard cells in stems, leaves and flowers. Upon stress treatment, LTI30 was detected in the vascular tissue of roots, stems, leaves, and flowers. LTI30 was also found in pollen sacks and generally distributed in roots.

Localization of DHNs in the vascular tissue in stressed plants is in agreement with previous reports (Bravo *et al.*, 1999; Danyluk *et al.*, 1994; Godoy *et al.*, 1994; Houde *et al.*, 1995; Schneider *et al.*, 1993). The presence of DHN-like proteins in root tips has been demonstrated previously in pea subjected to slow dehydration (Bracale *et al.*, 1997). The localization of LTI30 in the vascular tissue correlates to some extent with the results of Rouse *et al*. (1996). Promoter (*LTI30*) GUS-fusions showed staining in roots, desiccated pollen grains, trichomes and in the vascular tissue of leaves and stems (Rouse *et al.*, 1996). In unstressed plants, RAB18 staining was found in the nuclei of guard cells whereas after ABA treatment RAB18 was detected both in the cytosol and the nuclei in guard cells. Stress-induced promoter activity in stomatal guard cells has been reported for, *C. plantagineum DSP16* (Taylor *et al.*, 1995) and tomato *TAS14* (Parra *et al.*, 1996). Interestingly, both DSP16 and TAS14 are YSK-type DHNs,

as is RAB18. Two other studies report the accumulation of *DHN* mRNA in ABA-treated guard cells (Hey *et al.*, 1997; Shen *et al.*, 1995). The large body of information obtained in this study and the implications of the localization data are discussed further in the section, *proposed function of dehydrins*.

Ectopic expression of dehydrin genes in *Arabidopsis* (III)

In a more direct study to elucidate the contribution of DHNs to abiotic stress tolerance, we generated DHN overproducing Arabidopsis plants. We decided to overexpress multiple *DHN* genes in the same plant since previous attempts with single *DHN* genes in tobacco did not show significant improvements in tolerance compared to wild type plants (Iturriaga *et al.*, 1992; Kaye *et al.*, 1998; Lång, 1993). We have previously generated transgenic Arabidopsis overproducing RAB18 and LTI29 in which no increase in tolerance to drought and low temperatures was observed (Lång, 1993; Welin, 1994). Transgenic plants expressing either *LTI29* and *LTI30* (TP10) or *COR47* and *RAB1*8 (TP9) were made, and are herafter named SL29L30 and SC47R18. To compare our results obtained with these plants we also included transgenic Arabidopsis overexpressing *RAB18* (UVL82) alone, hereafter named SR18. All *DHN* genes were regulated by the constitutive *Cauliflower mosaic* virus 35S promoter, resulting in high accumulation of the proteins under non-stress conditions. Kanamycin-resistant plants were analyzed by western blot analysis to detect plants with high accumulation of the DHNs. Selected transgenic plants showing high DHN accumulation were thereafter subjected to salt, drought and freezing stress. Tolerance was compared with that of non-transformed wild type Arabidopsis and vector transformed control plants.

Effect of salt stress on cotyledon emergence

Transgenic Arabidopsis overexpressing DHNs showed an improved germination rate when placed on ½ MS medium complemented with 100 mM NaCl. Seed germination was enhanced 2-2.5 fold in all the DHN overproducing plants compared to control plants. At higher concentrations of NaCl (150 mM) germination was severely inhibited although a small fraction of seeds from some of the transgenic lines still produced viable cotyledons; SR18-3 (13%) and SL29L30-12 (4%). The improved salt tolerance effect was only seen during seed germination and subsequent cotyledon emergence, no such effect was detected when plantlets were transferred to high salt media after germination. Enhanced germination and cotyledon emergence were also observed when overproducing the barley DHN ABA2 in Arabidopsis (Calestani *et al.*, 1998). Salt tolerance during germination has been demonstrated for the *RS* (resistant to salt) and *rss* (resistant to salt stress) mutants, which have also been shown to be tolerant to osmotic stress (Saleki *et al.*, 1993; Werner and Finkelstein, 1995). As in the DHN overproducers, *RS* and *rss* mutants are not more tolerant than wild type at subsequent developmental stages. The *rss* mutation is suggested to be in the

sensing or signaling pathways following stress and the *RS* mutation, which mainly confers osmotolerance is also suggested to be involved in the signaling system (Saleki *et al.*, 1993; Werner and Finkelstein, 1995).

Overproduction of dehydrins results in enhanced drought tolerance

An even more remarkable phenotype of the DHN overproducing Arabidopsis was found to be associated with severe drought stress of whole plants in the greenhouse. When plants were allowed to dry out by withholding watering for ten days, wild-type plants and vector-transformed transgenic control plants all died, whereas DHN overproducers recovered upon rehydration. The best recovery was observed for SC47R18-2 (64%), SC47R18-3 (44%), SL29L30-12 (38%), SL29L30-18 (25%) and SR18-3 (19%). A similar result was obtained when a barley LEA (III) gene (*HVA1*) was ectopically expressed in rice. Plants wilted slower and less severely under drought in comparison to the wild type and upon rehydration the transgenic plants showed better recovery and resumed growth faster than wild type (Xu *et al.*, 1996). Previous attempts to assess the contribution of DHNs to drought tolerance by overproducing single DHNs have failed. Iturriaga *et al.* (1992) overproduced a *C. plantagineum* DHN (DSP16) in tobacco and Lång *et al.* (1993) overproduced RAB18 in tobacco and Arabidopsis. Those studies could not detect any effect of DHN overproduction in drought tolerance, however they did not analyze recovery after drought stress, only effects during the stress.

Our results indicate that the double constructs are more effective than the single construct. For instance, the recovery of SC47R18-2 is three times that of RAB18. The results also indicate that the DHNs tested share some common function, even if they might be specialized for different tissues as suggested by our localization study (Table 3).

Enhanced freezing tolerance in dehydrin overproducers

In a third stress study we subjected the transgenic plants to freezing temperatures to assess the effect of high DHN production and tolerance to freezing stress. Tolerance assessment was done in two different ways, first we analyzed effects on electrolyte leakage from freeze injured plants and secondly we examined survival at the whole plant level after a freeze treatment. Our results showed no apparent difference in electrolyte leakage between control and transgenic plants when testing non-acclimated plants. This is in agreement with results from transgenic tobacco overproducing the spinach DHN CAP85, which did not show improved freezing tolerance (Kaye *et al.*, 1998). However, after cold-acclimation a small difference in electrolyte leakage was detected (Table 4). The LT_{50} values show that overproduction of RAB18 has no effect in either non- or cold-acclimated plants. As discussed previously, RAB18 accumulates after ABA-treatment and not after low temperature-treatment and might have a specific function during drought stress.

To assess the effect of freezing at the whole plant level, frost survival experiments were performed with non-acclimated and cold-acclimated plants

(LE, SC47R18-2 and SL29L30-18). Non-acclimated DHN overproducing plants subjected to -7ºC or -9ºC for 4 hours showed less damage and an improved recovery and regrowth after freezing. Furthermore, DHN overproducers regained their turgor earlier than the wild type after freezing. A frost survival experiment was also done with cold acclimated plants subjected to -10°C for 4 hours. No clear differences in damage or in regrowth were detectable between different overproducers and control plants in this test. We believe that the temperature (-10°C) used in this experiment was too high, and new experiments will be performed at lower temperatures.

Table 4. LT_{50} values (°C) of transgenic Arabidopsis.
Survival of plant material was determined by the ion leakage method.
Value for 100 % leakage was obtained by freezing samples in liquid N_2.

line	non acclimated	cold acclimated
LE	-4.3 ±0.5	-6.6 ±1.1
VC	-4.7 ±0.7	-7.2 ±1.2
SC47R18-3	-4.7 ±0.7	-8.6 ±1.1
SL29L30-12	-5.1 ±0.1	-9.2 ±1.2
Sl29L30-18	-5.2 ±0.4	-9.1 ±1.3
SR18-3	-4.2 ±0.1	-7.2 ±0.

Values were obtained from 4 independent experiments each including 1-3 measuring points.

Cloning and characterization of a dehydrin gene from *Physcomitrella patens* (IV)

One of the most valuable tools for studies of DHN function would be the generation of a *DHN* null mutant. However, the redundancy of the DHN gene family and the lack of an efficient gene disruption method make the generation of such a mutant a formidable task. In *Arabidopsis* for instance, there are six characterized *DHN* genes (Gilmour *et al.*, 1998; Kiyosue *et al.*, 1994; Lang and Palva, 1992; Rouse *et al.*, 1992; Welin *et al.*, 1994). Moreover, there is an EST clone, *PAP310* (X91920) and in the AtDB we found two additional *DHN* genes, encoding a K_2 and a K_1 DHN. Screening of the Arabidopsis T-DNA mutant pool might be possible but still at least nine individual mutants would be needed to be crossed for creation of a true null mutant.

The recent development of reverse genetics in *P. patens* has opened up the possibility to generate gene-specific mutations in this species. This discovery has given plant scientists a new tool to study plant protein functions, as many general plant processes are also present in this lower land plant. Unfortunately, there are no *DHNs* characterized in bryophytes so we decided to look for possible *DHN* genes in *P. patens*. Our aim was first to establish if such genes are part of bryophyte genomes and secondly to establish if they fulfill similar function as in higher plants. If they exist and if they form part of the molecular mechanism of

response to abiotic stress, this would open up the possibility of using *P. patens* to determine the physiological function of DHNs by generating moss mutants.

To clone possible *DHN* genes from *P. patens* we used the consensus-degenerate hybrid oligonucleotide primer (CODEHOP) approach described by Rose *et al.* (1998). By using degenerate primers directed towards the conserved K- and Y-segments from DHNs of higher plants we were able to amplify a DNA-fragment that was shown to encode several Y-segments and one K-segment. The definition of a DHN was originally based on dehydration-induced expression (Close *et al.*, 1989) and has since shifted to a sequence based definition mainly referring to the K-segment. The partial gene was therefore named *PpDHNA* (*P. patens dehydrin A*). Full-length cDNA was obtained by 5'/3' RACE-PCR using primers designed from the partial sequence. The full-length cDNA was found to encode a hydrophilic protein with a MW of 59.2 kDa and a pI of 5.6. DHNA is rich in glycine (19%), charged amino acids (24%) and lacks the amino acids cysteine and tryptophan, all in accordance with the description of DHNs from higher plants (Close, 1996; Close, 1997). It is composed of eleven repetitive blocks covering almost the entire protein. Each of the eleven repeats contains a Y-like segment. Of those segments, one is perfectly conserved with Y-segments from higher plants whereas four have one amino acid substitution in the second position of the consensus. The remaining Y-like repeats have two to three amino acid substitutions. In the C-terminal part, a K-segment with 60% identity to the consensus K-segment of higher plants is located. When searching the protein database with the entire DHNA polypeptide, the highest similarity was found to a moss protein previously isolated from *T. ruralis* (AF275946). The *T. ruralis* protein is thought to be involved in cellular dehydration tolerance and has been termed rehydrin, even though it contains a K-segment. *T. ruralis* is a desiccation tolerant bryophyte and utilize a tolerance strategy that combines a constitutive protection system and a rehydration recovery mechanism.

Southern blot analysis indicates that *DHNA* is present as a single gene in the *P. patens* genome. ABA-treated and control moss were analyzed for the presence of proteins immunologically related to DHNs. Antibodies against the Arabidopsis LTI29 protein detected an ABA-inducible protein of about 110 kDa. When using antibodies against the Arabidopsis LTI30 protein, we detected several bands, a 50 kDa protein upregulated by ABA but present in control samples, a 35 kDa protein induced by ABA and finally two proteins around 110 kDa also induced by ABA. The two bands detected around 110 kDa might represent the same protein after post-translational modification such as phosphorylation and could also be the same protein we detected with the LTI29 antibody. From this experiment we conclude that there are probably three DHN-like proteins present in *P. patens*, this is further supported by the fact that the detected proteins remained soluble upon boiling, a property previously ascribed to DHNs (Close *et al.*, 1989).

We analyzed the accumulation of the *DHNA* transcript and found responsiveness to ABA. Expression was detected as early as 0.5 h after treatment with 1 µM ABA and higher concentrations of ABA further enhanced expression of *DHNA.* Since ABA is known to increase in response to salt and drought stress

we expected to detect *DHNA* transcript after exposure to these stresses. *DHNA* transcripts did accumulate in response to both salt and drought stress, further supporting the observed ABA-induction. In air-dried plants, transcript levels increased after 14 h of stress. Following rehydration, transcript levels decreased rapidly and could only be detected after 0.5 h. In salt-treated plants, mRNA accumulation was detected 2 h after treatment.

In conclusion, we have characterized a salt- and drought-inducible *DHN* gene from *P. patens*. The DHNA protein contains some of the conserved segments found in DHNs of higher plants. The study of DHNs in lower plants such as mosses might shed some new light on DHN function. Lower plants have the advantage of containing less differentiated tissue than higher plants, and this could be an advantage when studying the function of DHNs in general.

Proposed function of dehydrins (I, II and III)

DHNs are generally thought to play an important protective role during plant cellular dehydration, although no direct evidence for such a biochemical role has been presented. Studies indicate that DHNs improve enzyme activity under conditions of low water activity (Rinne *et al.*, 1999), possess cryoprotective activity (Close, 1996; Houde *et al.*, 1995; Kazuoka and Oeda, 1994; Wisniewski *et al.*, 1999) and antifreeze activity (Wisniewski *et al.*, 1999). Baker *et al.* (1988) suggested that DHNs act by solvating cytosolic structures, particularly membranes and this is supported by the finding that WCOR410 appears to be associated with the plasma membrane and is proposed to act by preventing membrane destabilization during dehydrative conditions (Danyluk *et al.*, 1998). The consensus K-segment has been proposed to form an amphipatic α-helix (Dure, 1993), furthermore it has been proposed to form a class A amphipatic helix (Close, 1996). The class A α-helices in apolipoproteins associate with lipids and it has therefore been suggested that one role of the K-segment could be hydrophobic interactions with membranes and denatured proteins (Close, 1996).

The observed strong binding of the recombinant DHNs to the Cu- and Ni – column, suggested that Arabidopsis DHNs could be involved in metal binding *in vivo* (Mäntylä, 1997). Abiotic stresses include an oxidative stress component, and the formation of active oxygen species is enhanced in the presence of many metal ions. Consequently, binding of free metal ions could theoretically reduce the formation of active oxygen species in dehydrated plant cells. The active oxygen species causes damage to proteins, DNA and lipids (Richter and Schweizer, 1997). The accumulation of active oxygen species under water deficit stress originates mainly from a decline in CO_2 fixation, leading to higher leakage of electrons to O_2. Reduction of O_2 generates superoxide (O^{*-}_2) and hydrogen peroxide (H_2O_2). In the presence of a metal catalyst the hydroxyl radical (OH*) can be generated. Even though our *in vitro* metal binding tests failed due to aggregation, we cannot exclude that this binding does not occur *in vivo*. However two results obtained after the metal binding studies do not support this theory.

The immuno-histochemical localization in unstressed plants showed that LTI29 and ERD14 were localized in the root tip and vascular tissues, possibly representing a preformed stress protection response. If the main function of DHNs were to chelate metals and prevent the formation of active oxygen species, one would expect localization at actively photosynthesizing tissues. Secondly, overproduction of DHNs in *Arabidopsis* enhanced cotyledon emergence under NaCl stress, whereas no tolerance was observed under subsequent developmental stages. Detoxification of active oxygen species has been shown to increase salt tolerance of the *pst1* (photoaxutrophic salt tolerant 1) mutant (Tsugane *et al.*, 1999). If DHNs were involved in chelation of metal ions, thereby decreasing the formation of active oxygen species a similar result would be expected in our transgenic plants overexpressing DHNs. We therefore suggest that DHNs are not involved in metal chelation *in vivo*, at least not to the extent that there is a reduction in active oxygen species.

In our localization studies we found that several members of this protein family are produced in non-stressed plants. DHNs were found to be produced in the root tip (ERD14 and LTI29), vascular tissues (ERD14, LTI29 and RAB18) and stomatal guard cells (RAB18). The localization data indicate that DHNs could have multiple functions or that they are specialized for different growth conditions, tissues, cells and stresses. The vascular system and the apical meristems are crucial for plant growth and survival, and it therefore seems likely that the localization of DHNs in these parts of unstressed plants could either be a preformed stress defense system or indicate a role in water uptake and transport. The root tip accumulation of DHNs might promote water influx into the actively dividing parenchymal cells of the root meristem. Similarly, in cells surrounding the xylem vessels DHNs might function as water attractants during the transport of water from xylem vessels to sink tissue. As a possible explanation for these observations one can postulate that DHNs could act as regulators of the osmotic potential. Following similar reasoning, the localization of RAB18 in nuclei of stomatal guard cells could protect the nuclei when stomata are open and the cytoplasm has a high osmolarity. Upon stress treatment, DHNs are localized in most cells and would, according to this theory, slow down the dehydration of tissues under stress. However, taking into account previously reported results and our findings when overexpressing DHNs in Arabidopsis, it is more likely that this accumulation represents a preformed stress protection of sensitive parts of the plant, which is necessary for plant survival during and after a desiccation event. We should however not discard the possibility that DHNs have multiple functions and that DHNs as well as being important for cellular survival during stress, may play an osmoregulatory role in certain cell-types under non-stress conditions.

Overproduction of DHNs in *Arabidopsis* enhanced the tolerance to drought and low temperature stress and increased the germination rate under salt stress. No salt tolerance was observed in two-week old plants. Under prolonged drought stress and after the frost survival experiment, DHN overproducing plants showed better recovery and regrowth than control plants. The common factor in these

results is growth / development from a desiccated state. This type of stress is also part of normal plant development for certain tissues *e. g*. seed desiccation and pollen development. It is therefore interesting that DHNs have been detected in both these tissues. We propose that DHNs, through an unknown mechanism, protect cellular structures during dehydration and enable cellular survival by stabilization and preservation of macro structures, predominantly in the cytoplasm and nucleus of the plant cell. The localization of DHNs in the vascular tissue and root tip seems plausible if DHNs have a protective function during dehydrative stress. Protection of these tissues are of utmost importance for survival. As suggested previously, this localization might be a preformed stress response designed for protection of the most sensitive parts.

Several studies have shown that DHNs are localized in the vascular tissues in stressed plants (Bravo *et al.*, 1999; Danyluk *et al.*, 1994; Godoy *et al.*, 1994; Houde *et al.*, 1995; Schneider *et al.*, 1993). Furthermore, subcellular localization of DHNs indicates their presence primarily in the cytoplasm and nucleus (Asghar *et al.*, 1994; Bracale *et al.*, 1997; EgertonWarburton *et al.*, 1997; Goday *et al.*, 1994; Godoy *et al.*, 1994; Houde *et al.*, 1995; Wisniewski *et al.*, 1999). Recently, localization has also been detected in chloroplasts (Wisniewski *et al.*, 1999), in or associated with mitochondria (Borovskii *et al.*, 2000) and the endoplasmic reticulum (Neven *et al.*, 1993). Furthermore, WCOR410 has been shown to be associated with the plasma membrane (Danyluk *et al.*, 1998). DHNs in birch have been shown to be localized in storage protein bodies and starch-rich amyloplasts (Rinne *et al.*, 1999), and in maize DHNs have been found associated to protein and lipid bodies (EgertonWarburton *et al.*, 1997). The widespread distribution of DHNs could be due to the fact that, as we propose, DHNs protect the stability of cells and their components. Two independent studies support our theory. In cowpea, studies have been made on varieties either containing or lacking a 35 kDa DHN (Ismail *et al.*, 1999). Varieties producing the 35 kDa DHN showed and an enhanced chilling tolerance during seedling emergence not seen in any variety lacking this protein, however no difference in electrolyte leakage was observed indicating that this difference is not due to specific plasma membrane protection. In another study Blackman *et al*. (1995) analyzed the role of ABA-induced proteins in soybean seeds, ABA-treated immature axes had high levels of LEA-like proteins but the electrolyte leakage was higher than that from mature desiccation tolerant axes. The difference in electrolyte leakage observed indicated that the membrane was damaged despite the presence of LEA-like proteins, and the authors suggests that LEA-like proteins enhance the structural stability of the cytoplasm (Blackman *et al.*, 1995). Taken together, these studies lead us to believe that DHNs preserve cellular structures (proteins and lipids) during dehydrative stress. The effect of this protection is not seen during the stress, but becomes clear when analyzing the recovery after stress. The fact that DHNs appear to be localized in most cellular compartments further support this theory.

Conclusions

During my studies I have studied a specific group of proteins that accumulate in response to abiotic stress in plants, the DHNs. DHNs are believed to play an important protective role during water-deficit stress, however until now no clear results have been presented regarding their effect or function during stress. My contribution to this field is summarized below:

- Development of a purification scheme including a technique (IMAC) previously not used for purification of DHNs. Four recombinant Arabidopsis DHNs (RAB18, LTI29, LTI30 and COR47) were purified with this method. I have also shown that DHN-like proteins from maize, rye, winter-wheat, spring-wheat and barley can be purified with IMAC.

- Evaluation of the purified proteins globular structure by gel filtration analyzes showed that RAB18, LTI29, LTI30 and COR47 behave as oligomers under native conditions. However, gel filtration under denaturing conditions revealed that the DHNs tested are probably not oligomeric proteins and we suggest that the observed phenomenon instead is due to an extended flexible structure of these proteins.

- Extensive localization studies revealed that DHNs accumulate in specific tissues and cells in Arabidopsis under normal growth conditions. We detected DHNs in the root tip (ERD14 and LTI29), vascular tissues (ERD14, LTI29 and RAB18) and in stomatal guard cells (RAB18). Upon stress treatment we detected ERD14, LTI29 and RAB18 in most cells. However strongest staining was found in cells surrounding vascular tissues.

- We found that LTI30 is probably both transcriptionally and post-transcriptionally regulated. LTI30 is not present in unstressed plants and upon stress treatment accumulates mainly in vascular tissues and pollen sacks.

- Overproducing DHNs in transgenic *Arabidopsis* generated plants with improved drought and freezing survival as well as improved seed germination at high salinity, thus demonstrating for the first time a direct contribution of DHNs to abiotic stress tolerance.

- Finally I have cloned and characterized a *DHN*-like gene from the moss *P. patens*. This is the first DHN genetically characterized in a bryophyte. Analysis of the open reading frame revealed a protein (DHNA) sharing several characteristics with DHNs from higher plants, including several Y- and one K-segment. *DHNA* transcripts accumulated in response to salinity, water stress and ABA treatment suggesting a conserved function of DHNs in all plants.

Future perspectives

My thesis has added some pieces to the puzzle of the function of DHNs in plants. However, much research remains to be done in order to solve the precise function of DHNs and to elucidate their detailed biochemical role in the plant cell.

- In my opinion the generation of a *DHN* null mutant would be a valuable step for more adequate functional studies of DHNs. As mentioned before this is not an easy task but could be done as a joint effort between different “DHN” labs.

- Cloning of the DHN-like proteins detected in ABA-treated *P. patens* This can be done by screening a cDNA expression library or by immuno-precipitation with the LTI30 antibody. Alternatively, the *DHNA* can be used for screening of a cDNA or genomic library or for design of degenerate primers.

- Subcellular localization of the DHNs we analyzed might yield more clues on DHN function. The localization of all DHNs in Arabidopsis should be done thereby presenting a complete picture of the spatial distribution of DHNs in one species. The DHN overproducing plants should be included in such a study, both stressed and unstressed plants to analyze if the DHNs protect cellular structures.

- Several biochemical analyses could be done on purified DHNs, such as lipid binding analysis, measurement of the osmotic potential and structural studies.

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