Freie Universität Berlin

Characterization of a Low Temperature Inducible Protein (BN26)

Inaugural-Dissertation

to obtain the academic degree

Doctor rerum naturalium (Dr. rer. nat.)

Submitted to the Department of Biology, Chemistry and Pharmacy of Freie Universität Berlin

By

Engy AboBakr AbdelBaky Khalifa

From Cairo, Egypt

Berlin 2013

2009 to 2013

Supervised by Prof. Dr. Jürgen M. Schmitt

Institute for Biology–Plant Physiology

Department of Biology, Chemistry, Pharmacy

Freie Universität Berlin

1st Reviewer: Prof. Dr. Jürgen. M. Schmitt

2nd Reviewer: Prof. Dr. Rupert Mutzel

Date of defence:

07.06.2013

Bescheinigung

Erklärung

Hiermit versichere ich, dass ich die Dissertation mit dem Titel "Characterization of a Low Temperature Inducible Protein (BN26)" selbstständig und ohne unerlaubte Hilfsmittel angefertigt habe.

Berlin, den.....

Engy AboBakr AbdelBakly Khalifa

.

Acknowledgement

First of all, I would like to praise and thank my gracious and great God “ALLAH” who enables me to carry out this work.

My first indebtedness goes to Professor Dr. Jürgen M. Schmitt who gave me the opportunity to work “under his roof”. I would like to express my deepest thanks for his kind supervision, continuous encouragement and valuable comments, in addition to improving the writing and presentation of my thesis.

Further, I would like to thank Professor Dr. Rupert Mutzel for evaluating my thesis and technical advice to commenting and correcting the thesis and valuable advices.

All the best feelings and deepest thanks for all the members of Schmitt work group; especially Dr. Carsten Köhn for contributing many helpful suggestions, help and support on my work.

Moreover, there is my big thank for Mrs. Hildegard Köht for the organisation of all the things in the lab, the isolation of thylakoids and CPP, all chemicals and the preparation, and for the friendly atmosphere and good cooperation.

I especially like to thank Mrs. Sigrid Kimura for the great intensive help and support with the paperwork and formalities for the dissertation.

My heartfelt appreciation goes to AG Romeis especially Andrea Werner and Anja Liese for the great help and support.

Big thanks also to my colleagues in AG Schmitt.

I also want to thank my mother and daughter Salma who have supported me the whole time and my neighbour Hussein and his family for the great support they gave me.

Great thanks to the Egyptian Government for the financial support offered to me to undertake this study in Germany.

Table of Contents

3.2 List of figures

II List of Tables

III List of Abbreviations

AP	Alkaline Phosphatase
APS	Ammoniumperoxodisulfate
ATP	Adenosin-5'-triphosphat
BMGH	Buffered Glycerol-Minimal Medium
BMMH	Buffered Methanol-Minimal Medium
BSA	Bovine Serum Albumin
bp	Basepare
°C	Grad Celsius
CF	Carboxyfluorescein
COR	Cold responsive
CD	Circular dichroism
CPP	Cryoprotectin
DNA	Desoxyribonucleicacid
dNTP	Desoxyribonukleosidtriphosphat
DTT	1.4-Dithiothreitol
DGDG	Monogalactosyldiaacylglycerol
E. coli	Escherichia coli
EDTA	Ethylendiaminotetraaceticacid
EPG	Egg Phosphatidylglycerol
EtOH	Ethanol
et al	And others
Epp.	Eppendorf
F	Fluorescence
Fig	Figure
h	hour
kb	Kilobasepare
KDa	Kilodalton
L	Liter
mA	Milliampere
mM	Millimolar
M	Molar
mg	Milligram
µg	Microgramm
min	Minute/Minutes
ml	Milliliter
µl	Microliter
µM	Micromolar
mRNA	Messenger RNA
nm	Nanometer
No	Number
NBT	p-Nitrotetrazoliumblauchlorid
N-Terminal	Aminoterminal
OD	Optical Denisty

ON	Over Night
PAGE	Polyacrylamid gel electrophoresis
P.s.	Pseudomonas syringae
pI	Isoelectric Point
P. pastoris	Pichia pastoris
rpm	Revolutions per Minute
RT	Room Temperature
SQDG	Sulfoquinovosyldiacylglycerol
Tab	Table
TEMED	N, N, N', N'- Tetramethylehylenediamine
TFA	Trifluoraceticacid
Tris	2-Amino-2-(hydroxymethyl)-1.3- propandiol
U	Units
UV	Ultraviolett
V	Volt
v/v	Volume per Volume
w/v	Weight per Volume
YNB	Yeast Nitrogen Base
YPD	Yeast extract-Pepton-Dextrose
%	Percent

1 Introduction

1.1 An overview over biological stresses and crop yield

Biological stress, can be defined as the force which inhibits normal functioning and well being of biological system as plants and animals (Jones and Jones, 1989). Nature's wrath in the form of various abiotic and biotic stresses adversely affects and limits plant growth and productivity (Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). Cold, heat, salinity and drought are among the major abiotic stresses which cause reduction in crop productivity and crop failure (Bray *et al.*, 2000; Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006). Plants differ from animals in that they are sessile. As plants are sessile, they adapt to various stresses by developing different mechanisms that enable them to elicit complex interactions between signalling molecules and pathways (Xin and Browse, 2000; Smallwood and Bowles, 2002; Mahajan and Tuteja, 2005; Martin *et al.*, 2012). Fig.1 summarizes these adaptations of plants to sense abiotic stresses such as cold, drought and salinity.

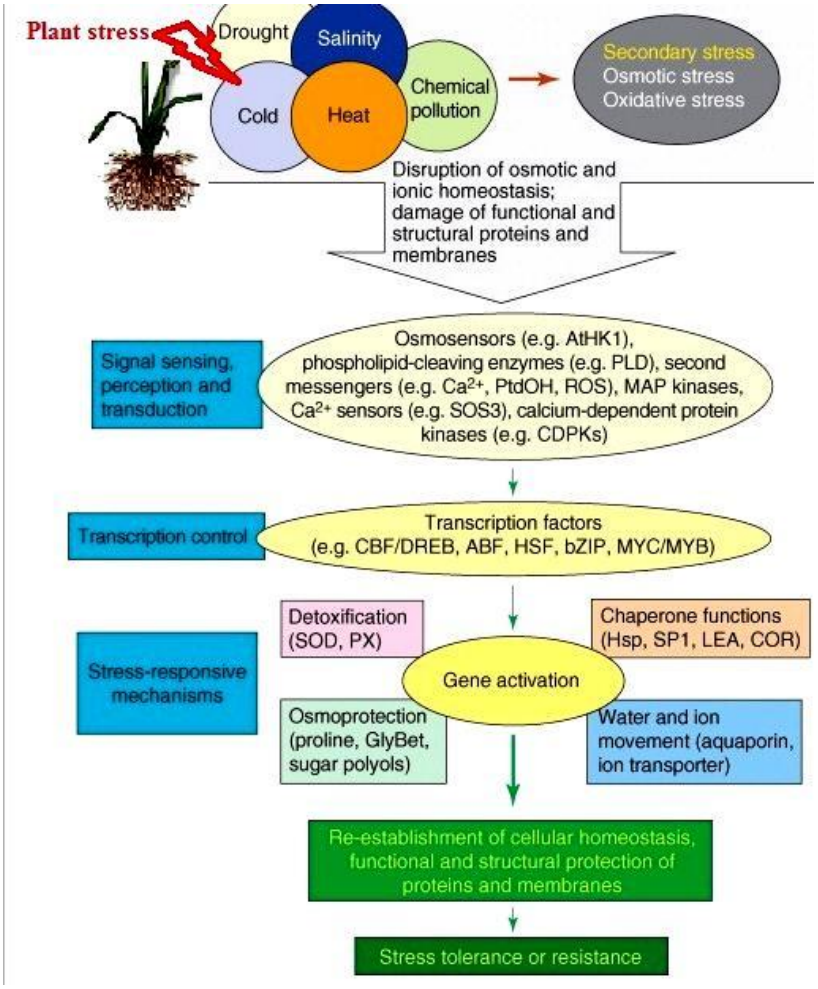


Figure 1: Diagrammatic representation of plant response pathway to primary abiotic stresses such as salinity, drought and cold stress from (Vinocur and Altman, 2005). Stress imposes injury on cellular physiology and results in metabolic disfunction and membrane fluidity. This results in a negative influence on cell division and growth of a plant (Levitt, 1980; Palta, 1989; Mahajan and Tuteja, 2005). Abbreviations: ABF, ABRE binding factor; AtHK1, *Arabidopsis thaliana* histidine kinase-1; bZIP, basic leucine zipper transcription factor; CBF/DREB, C-repeat-binding factor/dehydration-responsive binding protein; CDPK, calcium-dependent protein kinase; COR, cold-responsive protein; Hsp, heat shock protein; LEA, late embryogenesis abundant; MAP, mitogen-activated protein; PLD, phospholipase D; PtdOH, phosphatidic acid; PX, peroxidase; ROS, reactive oxygen species; SOD, superoxide dismutase; SP1, stable protein 1.

1.1.1 Cold stress of plants

Cold stress is a major environmental factor that limits the agricultural productivity of plants and causes yearly crop losses (Boyer, 1982; Chen, 1994; Xin and Browse, 2000; Pearce, 2001; Yamaguchi-Shinozaki and Shinozaki, 2006). According to their respective cold responsiveness plants are divided into three groups. The first group is susceptible and damaged by temperatures below 12 °C whereas the second group is able to acclimate to temperatures below 12 °C but unable to survive freezing, and the last group is freeze-tolerant and able to acclimate to survive temperatures significantly below freezing (Pearce, 1999; Thomashow,1999; Sharma *et al.*, 2005). However, the actual degree of tolerance is dependent on the species, developmental stage and duration of cold stress.

1.1.1.1 Freezing stress

Cold temperature causes a great effect on cellular components and metabolism. Maximov, 1912 hypothesized that the plasma membrane is the primary site of freezing injury. Previous studies indicated also that the cell membrane system is the primary site of freezing damage and much research was done on the biochemical and physical properties of membranes and the effects of freezing on membrane structure (Gordon-Kamm and Steponkus, 1984; Steponkus, 1984; Uemura and Yoshida, 1984; Lynch and Steponkus, 1987; Palta, 1989; Uemura and Steponkus, 1994).

1.1.1.1.1 Freeze-induced dehydration

When temperature drops below 0ºC, freezing injury starts by ice crystals formation in the intercellular spaces (Burke *et al.*, 1976; Chen, 1994; Browse and Xin, 2001; Jan *et al.*, 2009). The formation of ice crystals leads to decrease the water potential outside the cell and osmosis force water out (Levitt, 1980; Webb and Steponkus, 1993). The dehydrating effect of extracellular ice dominates the biophysical effects of sub-zero temperature on cell membranes.

This freeze-induced dehydration causes profound effects resulting from cell-desiccation increase (Burke *et al.*, 1976; Levitt, 1980; Guy, 1990). This desiccation results at the end of cellular lethal damage due to denaturation and precipitation of proteins and other molecules (Steponkus and Webb, 1992; Webb and Steponkus, 1993; Thomashow, 1998; Pearce, 2001). However, the plasma membrane as the primary site of injury shows that injury occurs at the membrane level (Steponkus, 1984; Steponkus and Webb, 1992). Therefore, as long as the plasma membrane remains intact the ice is confined to the outside of the cell (Levitt, 1980). A decrease in temperature rigidifies the cell membrane, as the membrane fluidity or viscosity is directly and reversibly affected by changes in temperature.

Fig. 2 illustrates the movement of unfrozen water down the chemical potential gradient from inside the cell to the intercellular spaces (Steponkus, 1984; Smallwood and Bowles, 2002; Sharma *et al.,* 2005; Jan *et al.*, 2009).

The thermal damage on bilayers occurs as consequence of freeze induced cellular dehydration including expansion-induced lysis, lamellar-to-hexagonal-II phase transitions (Fig. 3), and fracture jump lesions (Palta, 1989; Steponkus *et al.*, 1993b; Webb *et al.*, 1994). Membrane lipids, in non-acclimated tissues undergo lateral phase separations and form lamellar-to-hexagonal-II-phase transitions in regions where the plasma membrane is brought into close apposition with subtending endomembranes, which leads to destabilization of the plasma membrane and ion leakage (Steponkus *et al.*, 1990; Steponkus and Webb, 1992). It has been shown by various experiments that freezing-induced destabilization of the plasma membrane involves different types of lesions which are depending on the stage of cold acclimation and the extent of freeze-induced dehydration (Steponkus, 1984; Steponkus *et al.*, 1993a; Webb *et al.*, 1994; Uemura *et al.*, 1995).

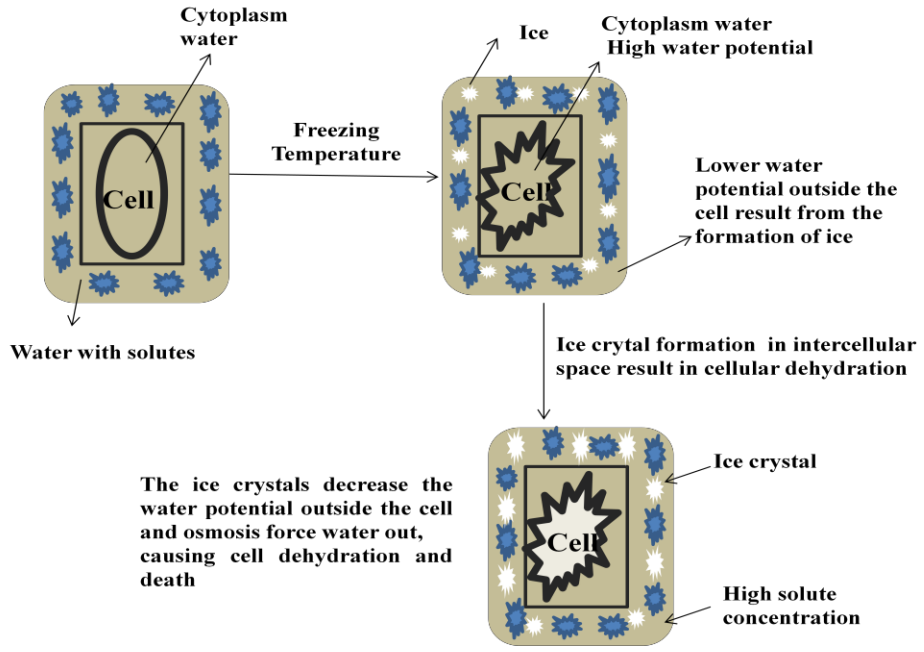


Figure 2: Diagrammatic representation showing the mechanism of cellular dehydration at freezing temperature from (Sharma *et al.*, 2005; Jan *et al.*, 2009). The formation of intercellular ice is the major reason behind cellular dehydration at low temperature (Smallwood and Bowels, 2002). During extracellular freezing, ice forms between cells, which in turn initiate the removal of free water from cells which leads to dehydrative stress in the cells (Uemura and Steponkus, 1999).

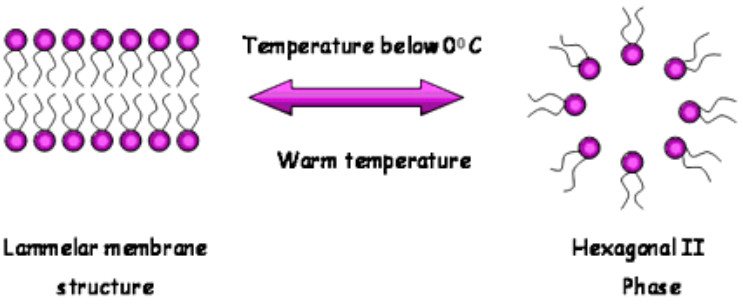


Figure 3: Digrammatic representation showing the transition of membrane structure from lamellar to hexognal II phase at freezing temperature and vice versa at warm temperature from (Jan *et al.*, 2009). In hexagonal II phase lipids are not in bilayer but they form long tubes (Steponkus, 1984; Steponkus *et al.*, 1993b; Oliver *et al.*, 2002).

1.2 Cold acclimation

In the 1950's the term "cold acclimation" has been originally used by animal physiologists to describe physiological changes in organisms when exposed to low non-freezing temperature. In the 1960's, the term used for plants was defined as the process of plant adjustment to low temperature (2 ºC) which occurs to varying degrees in hardy and non-hardy varieties (Kenefick, 1963). Kacperska (1989) has reported that the cold acclimation of biennial plants leads to two distinct effects, namely the improvement of the performance of cells at low temperature and increased resistance to freezing. In the last years there is a big challenge to plant physiologists and molecular biologists in the separation of global metabolic adjustments to low nonfreezing temperature and the processes involved in the induction of freezing tolerance.

Cold acclimation or overwintering is simply a term used to describe the transition from tender to hardy status (Chen, 1994). Cold acclimation results in a number of morphological, physical and biochemical changes of cell membrane fluidity through changes in the lipid composition and induction of non-enzymatic proteins that alter the freezing point (Levitt, 1980; Thomashow, 2001; Jan *et al.*, 2009). The plant cells undergo biochemical, molecular, physical and structural rearrangements in their macromolecules to tolerate freezing induced cellular damage during winter (Fig. 4). Some of the most frequently described metabolites that contribute to freezing tolerance are soluble sugars, proline, increased levels of antioxidants, the modulation of activities of various enzymes, the production of dehydrins, osmotins, antifreeze proteins (AFPs), and chaperones which stabilize and maintain correct conformation of proteins and RNAs (Thomashow, 1990; Griffith *et al.*, 2005; Shinozaki and Yamaguchi-Shinozaki, 2007). According to Steponkus *et al.*, (1993) cold acclimation increases freezing tolerance in rye and other plants by preventing expansion-induced-lyses and the formation of hexagonal II phase lipids in the membranes.

Reduction in membrane fluidity seems to act as a sensor to low temperature effect and this rigidification can be sensed and might represent a potential site of perception and/or injury (Horvath *et al.*, 1998; Örvar *et al.*, 2000; Knight and Knight, 2012). Membrane rigidification effect is thought to activate temperature sensors located in the plasma membrane. The close relationship of the plant cytoskeleton with the plasma membrane, the major platform for signal perception and transduction (Wasteneys and Galway, 2003).

The plant cytoskeleton has a central role in cold signalling and acclimation as the alteration of cytoskeleton configuration which potentially resulting in a modified Ca^{2+} signature (Örvar *et al.*, 2000, Sangwan *et al.*, 2001). At the early phase of cold acclimation, flexibility and re-organization of the microtubule cytoskeleton is needed, while completion of the process requires the formation of cold-stable microtubules, which ensure growth even when the temperature is not optimal (Fisher *et al.*, 1996; Wasteney and Galway, 2003). This suggests that microtubules and microfilaments are downstream targets of various signalling pathways (Wasteney and Galway, 2003).

Cytoskeletal reorganization serves as a link between membrane rigidification and Ca^{2+} ions influx through the plasma membrane and release from the vacuole (Knight *et al.* 1996; Örvar *et al.*, 2000; Sangwan *et al.*, 2001, 2002). This is because cytoskeleton reorganization leads to opening of the Ca^{2+} channels and subsequent Ca^{2+} influx. The increased $[Ca^{2+}]$ is then used as a signal for cold acclimation response (Sangwan *et al.*, 2001, 2002).

Transient elevation of intracellular calcium is considered to be an early event in a plant cell's response to low temperature (Knight *et al.*, 1991). The elevation of the cytosolic Ca^{2+} concentration is characteristic for the response to various abiotic and biotic stimuli (Knight and Knight, 2000). A critical role of calcium (Ca^{2+}) as the most common signal transduction element in the cells emerged for the process of sensing and transduction (Knight and Knight, 2000).

Moreover, the acclimation is triggered by a Ca^{2+} influx into the cytosol, which is a requirement for the induction of *cor* genes (Knight *et al.*, 1991; Monroy and Dhindsa, 1995; Knight *et al.*, 1996). One of the key roles of Ca^{2+} induced cellular metabolites is to stabilize cell membranes against freezing injury during the winter period. Steponkus and Lynch (1989) showed that such stabilization of membranes is a result of osmotic adjustment and the accumulation of solutes such as sucrose and proline.

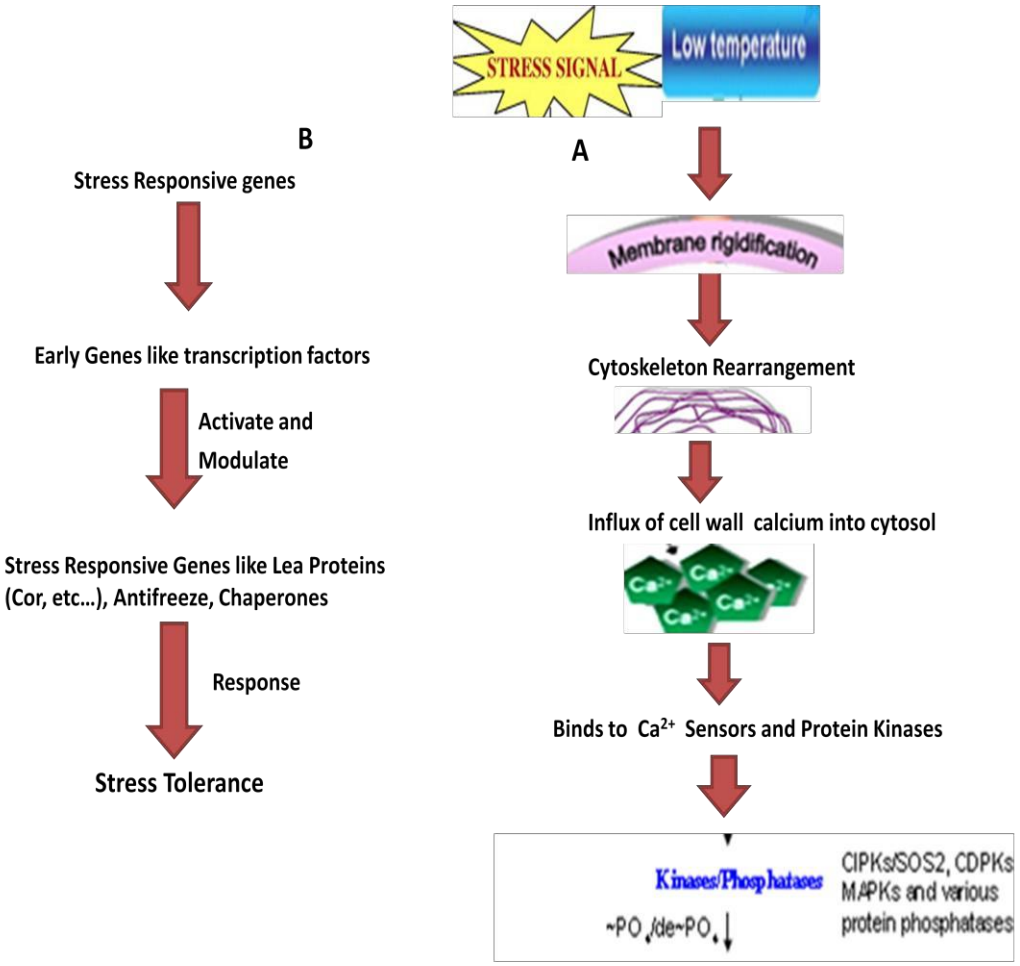


Figure 4: Schematic representation of early (A) and late events (B) in cold response in higher plants adapted from (Mahajan and Tuteja, 2005). (A) There is evidence that cold is sensed via changes in plasma membrane fluidity (Martinieire *et al.*, 2011) leading to Ca^{2+} influx and release from the vacuole (Örvar *et al.*, 2000). (B) Cold acclimation involves precise regulation of expression of transcription factors and stress responsive effecter genes like cold-regulated (COR) genes (Thomashow, 1999; Seki *et al.*, 2001, Seki *et al.*, 2002; Kreps *et al.*, 2002; Xiong *et al.*, 2002b; Shinozaki *et al.*, 2003).

1.2.1 Cold responsive genes

1.2.1.1 Regulation of gene expression in response to low temperature

In the 1970's, it was proposed that cold acclimation involves alterations in gene expression (Weiser, 1970). Analysis of the expression profiles of cold-inducible genes during low temperature treatment revealed the existence of at least two groups that have different temporal patterns of expression (Fowler and Thomashow, 2002; Seki *et al.*, 2002). In the first group, the expression is rapid and transient in response to low temperature encoding components of the signal transduction pathways, and in the second, the expression increases gradually during cold treatment and these genes directly participate in cell protection (Fowler and Thomashow, 2002; Seki *et al.*, 2002; Heino and Palva, 2003; Vogel *et al.*, 2005; Van Buskirk and Thomashow, 2006). Fowler and Thomashow (2002) showed that in *Arabidopsis* nearly 30% of cold-regulated genes were down-regulated during acclimation, encoding proteins involved in transcription, signaling, cell-wall biogenesis, and defense. One important group of down regulated genes was those involved in photosynthesis (Fowler and Thomashow, 2002).

Cold-inducible genes are relatively diverse in sequence and form distinct groups regarding similarity in their amino acid sequence. However, many of them share common properties, such as being extremely hydrophilic, resistant to heat denaturation and composed largely of repeated amino acid sequence motifs. These properties are thought to enable them to protect cells against freezing injuries by stabilizing both proteins and membranes during cold stress. Cold acclimation is a genetically controlled trait induced by low temperature. Polypeptides induced by low temperature have been reported in Alfalfa (Mohapatra *et al.*, 1987), *Arabidopsis* (Lång *et al.*, 1989), and potato (Lee *et al.*, 1992).

The ability of plants to acquire freezing tolerance from cold acclimation has been shown to involve the reprogramming of gene expression networks (Seki *et al.*, 2001, Fowler and Thomashow, 2002; Fowler and Thomashow, 2002; Kreps *et al.*, 2002; Seki *et al.*, 2002; Heino and Palva, 2003).

1.2.1.2 Localization of cold responsive proteins

Very little is known about the distribution of low temperature inducible proteins. Several acclimation-specific proteins were found in leaf and stem of spinach but not in the roots. Similarly, Perras and Sarhan, 1989 identified several proteins that occur only in leaves. Sub-cellular distribution of low temperature inducible proteins revealed that some are in the chloroplast (Lin and Thomashow, 1992b). Others are in the nucleus (Houde *et al.*, 1995), endoplasmic reticulum and cytosol (Neven *et al.*, 1993).

1.2.1.3 Evolution and adapative duplications in genes in *Brassica* (Brassicaceae)

An old world genus, Brassicaceae, 50 million years (Ma) ago probably appeared and the main lineages of the family had diverged (Koch *et al.*, 2000). Crucifers *Brassica* includes about 35 species of mostly annual herbs, with some perennial herbs and small shrubs. The hybridization of *B. oleracea* and *B. rapa* to form the *B. napus* genome, most probably occuring less than 10,000 years ago (Al-Shehbaz *et al.*, 2006). Figure 5 shows that *Brassica napus* (L.) is an amphidiploid species that originated from a spontaneous hybridization of *Brassica rapa* (L.) and *Brassica oleracea* (L.), and contains the complete diploid chromosome sets of both paternal genomes (Snowdon *et al.*, 2002).

For these cruciferous plants the induction of alanine-rich proteins under abiotic stress could be shown (Baimiev *et al.*, 1999; Gimalov *et al.*, 2001). One of these alanine-rich proteins functionally characterized in abiotic stress resistant in Arabidopsis is Cold-Regulated15A (COR15A) (Artus *et al.*, 1996). For COR15A three homolog proteins could be identified in the taxon Brassica probably originating through evolution by gene duplication events. In evolutionary innovations gene duplication has long been considered to play an important role (Ohno, 1970; Walsh, 1995). Cavell *et al.*, (1998) showed that *Arabidopsis* is approximately 87% similar to *B. oleracea* at the sequence level in exons.

Three homologues genes to *cor15*, a gene with descript function in *Arabidopsis* cold stress response; have been discovered in the species *Brassica napus* (*bn26, bn115*, and *bn19*) which are also involved in responses to cold stresses (Weretilnyk *et al.*, 1993). In my study we have cloned the homologue of BN26 from *Brassica oleracea* (L.) and expressed it in *Pichia pastoris.*

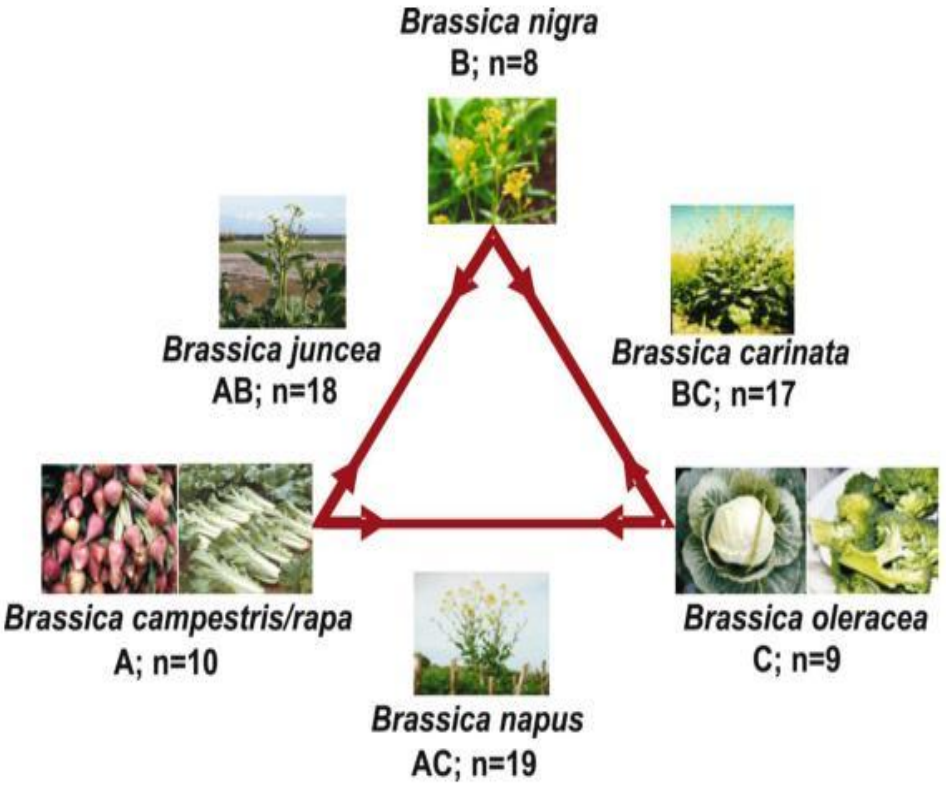


Figure 5: The U-triangle showing derivation of the high chromosome *Brassica* species from low chromosome species according to (U, 1935). Cultivated brassicas are represented by six interrelated species, three of which are diploids *Brassica nigra (2n = 16,* genome BB), *B. oleracea (2n = 18, genome CC), and Brassica rapa (2n = 20,* AA) and three amphidiploid derivatives which are: *B. carinata (2n = 34, BBCC), B. juncea* (2*n = 36, AABB), and B. napus (2n = 38, AACC).* The latter three are derived by hybridization and polyploidization of two of the diploid taxa through each possible pair-wise combination (Warwick and Al-Shehbaz, 2006).

1.2.1.4 Cold responsive genes with known function

(1) Cryoprotectin

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 a freeze-thaw cycle to -25 °C. Later it was demonstrated that this cryoprotective activity is in fact 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). Tab. 1 hydrophilic polypeptides encoded by COR genes that are induced during cold acclimation and their homologues with unknown function

Table 7: Hydrophilic polypeptides encoded by COR genes that are induced during cold acclimation and their homologues with unknown function (Thomashow, 1998).

Table 1. *Hydrophilic polypeptides encoded by COR genes that are induced during cold acclimation*

Group	Plant	Polypeptide	Accession No.[a]
COR6.6	Arabidopsis	COR6.6/KIN2	X55053/X62281
		KIN1	X51474
	Brassica napus	Kin1	M81224
	Brassica rapa	Kin	L31939
COR15a	Arabidopsis	COR15a	X64138
		COR15b	L24070
	B. napus	BN115	541910
		BN26	541912
		BN19	541911
	Brassica oleracea	BoCOR	U16751
COR78	Arabidopsis	COR78/LTI78/ RD29a	L22567/X67671/ D13044
		LTI65/RD29b	X67670/D13044
	Spinacia oleracea	CAP160	AF035535
WCS19	*Triticum aestivum*	WCS19	481813
	Hordeum vulgare	CR1	117309
CORa	*Medicago sativa*	CORa	L03708
		ESIPa	M74190
	Medicago falcata	GRPa	X59930
CAS15a	*M. sativa*	CAS15a	L12461
		CAS15b	L12462
COR47 (LEA II)	Arabidopsis	COR47/RD17	X90959/ AB004872
		LTI29/ERD10	X90958/D17714
		LTI30/XER02	X77613/U19536
		ERD14	D17715
		RAB18	282880
	H. vulgare	DHN5	M95810
	M. falcata	CAS18	L07516
		CAS17	L13415
	Poncirus trifoliata	COR19	2147192
		COR11	2147191
	Prunus persica	PCA60	U62486
	Solanum tuberosum	CI7	U69633
	S. oleracea	CAP85	M96259
	T. aestivum	WCS120	M93342
		COR39	445603
		WCS66	L27516
		WCOR726	U73213
		WCOR80	U73212
		COR410	1169018
	Vaccinium corymbosum	BBDHN1	AF030180
HVA1 (LEA III)	*H. vulgare*	HVA1	X78205
	T. aestivum	WCOR615	U73217

[a] Accession numbers are for nucleic acid (those beginning with letters) and protein (those without letters) databases.

(2) COR6.6, COR15, BN26 and BN115

It was observed that often the same genes are expressed in plants when they are exposed to cold, drought or salt, most probably because all these stresses lead to physiological drought and the main function of the encoded proteins is to protect the cells from cellular dehydration under all these stress conditions (Knight *et al.*, 2001; Knight *et al.*, 2004).

Genes encoding for a number of cold regulated proteins (COR) have been isolated from *Arabidopsis thaliana*. Analysis of COR6.6 showed that the peptide is hydrophilic and alanine-rich (Gilmour *et al.*, 1996). It has 96% amino acid homology with KIN1 and is a cytoplasmic 6.6 kDa polypeptide. kin1 is induced by water stress and the plant hormone abscisic acid (ABA) which has been suggested to be a common mediator for osmotic stress responses and cold acclimation in plants (Kurkela and Franck,1990).

COR15A was first isolated from *Arabidopsis thaliana* of the Brassicaceae, encoding a 15 kDa protein with substantial similarities in its amino acid sequence to those encoded by LEA (late embryo abundant protein) genes (Lin and Thomashow, 1992). This protein is located in the stromal compartments of chloroplasts and is involved in the dehydration tolerance mechanisms of cold-stressed plants. COR15A is a helical amphipathic polypeptide localized in the chloroplast stroma. Over-expression of the *cor15* gene can reduce the propensity of membranes to form hexagonal-phase lipids during freezing stress (Steponkus *et al.*, 1998) and enhance the cold-tolerance. Even more also in other species over-expression of COR15A lead to a enhanced cold-tolerance (Artus *et al.*, 1996).

White *et al.*, (1994) isolated one of three COR15A homologues in *Brassica napus*, BN115. Analysis of upstream regions of BN115 identified two 8-bp elements which:

I. Are identical to positive regulatory regions of the promoter of the *cor15* gene.
II. Contain a 5-bp core sequence of low-temperature and dehydration responsive elements in promoter regions of several cold responsive *Arabidopsis thaliana* genes.

Promoter regions of *Cor*/*Lea* genes commonly contain a CCGAC core motif called CRT (C-repeat)/DRE (dehydration responsive element) in *Arabidopsis* (Yamaguchi-Shinozaki, 1994; Bakr *et al.*, 1994).

Currently nothing is known about BN26 and its possible role in low temperature acclimation. However, promoter analysis of *bn26* gene using PLACE database reveals the presence of *cis*-acting elements which can be attributed to abiotic stress induction of the gene. The best characterized of these is the dehydration-responsive element (DRE), also known as a C-repeat (CRT) or a low-temperature-responsive element (LTRE). Tab. 2 shows a comparison between BN26 and COR15A

Table 8: Comparison between BN26 and COR15A

	COR15A	BN26
Hydrophilicity	+	+
Theoretical pI	4.5	4.2
Amino acid Composition	rich in alanine, lysine, glutamic acid and aspartic acid residues which comprise more than 60% of the protein and devoid of Pro, Met, Trp, Cys, Arg, Gln	rich in alanine, lysine, glutamic acid aspartic acid residues which make up more than 50% of the protein and devoid of Pro, Trp, Cys, His,and Phe
% identity	69%	100%
localization	Chloroplast	Predicted to be chloroplast protein

BN26 is one of the homologues of COR15A with unknown function. Three closely related genes have been identified in *Brassica napus* BN115, BN26 and BN19 (Weretilnyk *et al.*, 1993). The objectives of this thesis were to gain insight into the role of BN26 during low temperature acclimation by a combination of biophysical and biochemical studies. The identity between BN26 isolated from *Brassica oleracea* and that from *Brassica napus* is 99% which means that the two genes are almost identical. The identity between COR15A and BN26 is 69% (Fig. 6).

(A)

```
BN26-6   AEKGDGNILDDINEATKRASDYVTDKTKEALKDGEKAKDYVDEKNDEAKDTALDEAQKVL
BN26     AEKGDGNILDDINEATKRASDYVTDKTKEALKDGEKAKDYVDEKNVEAKDTALDEAQKVL
Expasy   ************************************************************

BN26-6  DYVKEKGNEAGEAKDTTKA
BN26    DYVKEKGNEAGEDKDTTKA
Expasy  ********** ******
```

(B)

```
BN26     MAMSFSGAVLSGINSSFPSGVAKKSGVAKQSGVGAVRFGRKTELVVVAQRKKSLIYAEKG
COR15A   MAMSFSGAVLTGMASSFHSG-------AKQSSFGAVRVGQKTQFVVVSQRKKSLIYAAKG
         **********:*: *** **        ****..****.*:**::***:********* **

BN26     DGNILDDINEATKRASDYVTDKTKEALKDGEKAKDYVDEKNVEAKDTALDEAQKVLDYVK
COR15A   DGNILDDLNEATKKASDFVTDKTKEALADGEKAKDYVVEKNSETADTLGKEAEKAAAYVE
         *******:*****:***:********* ********* *** *: **  .**:*.  **:

BN26     EKGNE-----------AGEDKDTTKA
COR15A   EKGKEAANKAAEFAEGKAGEAKDATK-
         ***:*           *** **:**
```

Figure 6: (A) Alignment between BN26-6 (*Brassica oleracea*) and BN26 expasy (*Brassica napus*). The Blast and Clustal W/X were used to multialign and screen the amino acid sequence similarity through GenBank database (http://www.ncbi.nih.gov) without signal peptide. (B) Alignment between BN26 and COR15A amino acid sequence. Identical amino acids are highlighted in yellow. Highly homolog and homolog amino acids are highlighted in lila and turquose, respectively.

1.3 Aim of the study

Various approaches in the recent years have been done to enlighten the function of low-temperature inducible proteins. BN26 is a homologue of the *Arabidopsis* well characterized polypeptide COR15A with unknown function. The purposes of this thesis were to investigate functional and structural properties of BN26. For this reason, *in vitro* experiments were planned to reveal the interaction of the BN26 protein with both artificial membranes and proteins. For protein protection assays, distinguishing between either protection during denaturation under freezing and dehydrative stresses or refolding (chaperone) activity was performed.

Due to the lack of functional studies concerning BN26, *in vitro* studies were performed in order to detect a possible structure-function relationship. For this reason, an estimation of the secondary structure of BN26 protein should be performed by circular dichroism spectroscopy

I therefore, addressed the following questions:

- Does BN26 have an unordered native structure?

- Does BN26 have any effects on the cryostability of liposomes *in vitro*?

- Does BN26 protect thylakoid membranes against freeze-thaw damage and could this be considered as a possible mechanism of freezing tolerance?

- Does BN26 have chaperone activity?

- Does unstructured proteins could protect other proteins from denaturation during freezing and dehydration stresses?

- Does an unstructured protein could protect other proteins from denaturation during freezing and dehydration stresses?

The results from functional and structural characterization of BN26 can give an insight in the mechanism by which BN26 could have a role in cold acclimation process and compare this mechanism of cryoprotection with that of the well-known cryoprotective protein COR15A.

3.1 Materials

3.1.1 Plant Material

3.1.9.1 Spinach

Spinach (*Spinacia oleracea* L. cv. Monnopa) was grown in a growth chamber from seeds obtained from Julius Wagner GmbH (Heidelberg, Germany). The plants were cultured in 50x30 cm large pots in a climate chamber at 25°C during the day and 15°C at night with a light regime of 14 hours light (150 µmol quanta m^{-2} s^{-1}) and 10 hours of darkness.

3.1.1.2 Cabbage

Seeds of *Brassica oleracea* (*convar. Capitata var. Sabauda L. cv. Tasmania)* plants were from the company *Syngenta* Agro GmbH (Maintal, Germany). Cabbage was planted in pots of 20 cm diameter in the climate chamber. The light cycle was 14 hours light and 10 hours darkness. The luminous intensity of the light bulb planting flora-F was from 2000 to 2500 Lux (150 µ mol quanta m^{-2} s^{-1}). After three weeks, the young plants were transplanted either in the outdoors and taken in winter after cold, or pre-transferred to a refrigerator at 5°C for 14 days. The leaves then were harvested and frozen for long-term storage at -20°C. Of these freeze-stressed plants, we were able to isolate the cryoprotectin protein with cryoprotective activity.

3.1.2 Chemicals

The chemicals used were obtained from the following companies:

Applichem (Darmstadt, Germany), Bio-Rad (München, Germany), GE Healthcare (Freiburg, Germany), Fluka (Buches, Schweiz), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) or Sigma (München, Germany), Roche (Mannheim, Germany).

3.1.3 Equipment

Columns	pharmacia fine chemicals
Microwave	Quelle, Germany
pH-Meter	Knick, Berlin
Protein-Gelelectrophoesis- Apparatus	Workshop FU-Berlin
Icemaker	Ziegra, Germany
Scales	(GT 480) OHAUS, U. S. A. (H110) Sartorius, Germany
Clean bench	Gelaire, Germany
Water baths (3042)	Köttermann, Germany
Vortex	IKA Labortechnik, Germany
Electophoresis Powersupply PHERO-stab. 300	Biotec-Fischer,Germany
Spectralphotometer (Novaspec® II) (Ultrospec II 4050)	Pharmacia LKB Biochrom., England
Centrifuges (Biofuge28S)	Hereaus Sepatech. Berlin, Germany

Tabletopcentrifuge5415C	Eppendorf, Germany
2K15 Refrigeratedcentrifuge	Sigma,Daisenhof
Thermostat (Blockthermostat BT 100)	Kleinfeld Labortechnik, Germany
Multiscan Thermosceintific Microtiter plate reader, 96- system	Thermo Fisher Scientific, Finland
Shaker (KS 10)	Edmund Bühler, Tübingen
Luminator	Berthold Technologies U.S.A.
Hand held extruder Lipids	Avestin, Ottawa, Canada

3.1.4 Consumables

Nitrocellulose membrane (Hybon™-extra)	AmershamBiosciences, Freiburg
His-Trap columns	GE-healthcare
Black 96-well microtiter plate	Promega
Transparent 96-well microtiter plate	Serva

3.1.5 Kits

EasyComp-Kits K1730-01 (Invitrogen)

3.1.6 Genes

	Acc. No. (NCIB)	Reference
COR15A (ABRC Clone U12858)	At2g42540	(Thomashow, 1994)
BN26 (*Brassica oleracea*)		provided by Carsten Köhn AG Schmitt
His-BN26 (*Brassica oleracea*)		constructed by Silke Schilling and provided byAG Schmitt
Pseudomonas syringae allantoicase (ALC) his-tagged protein expressed in *E.coli*		(Werner *et al.*, 2010) provided by AG Romeis
BN26: cold-induced protein from *Brassica napus* (canola)	AAB29484.1	(Weretilnyk *et al.*, 1993)

3.1.7 Antibodies

Antibody	Source
Polyclonal Anti-WAX9	Hincha *et al*., 2001
70-6515 Goat Anti-Rabbit IgG (H+L)-HRP	Bio-Rad
Monoclonal Anti-polyHistidine, Clone HIS-1 (H1029)	Sigma
Anti-Mouse IgG-Alkaline Phosphatase	Sigma

3.1.8 Enzymes

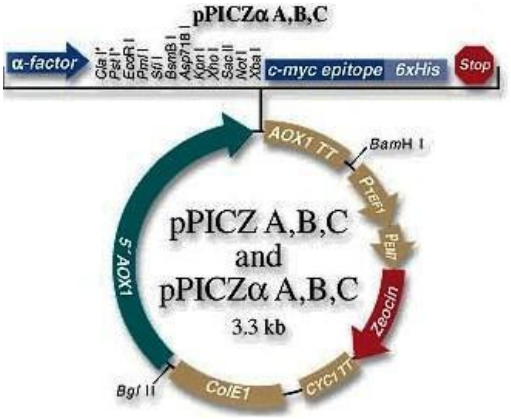
L-lactate dehydrogenase from rabbit muscle (EC 1.1.1.27)	Roche
Quantilum Recombinant Luciferase	Promega
Luciferase Assay System	Promega

3.1.9 Plasmids

3.1.9.1 Expression vector pPICZα

pPICZα (Invitrogen) contains the *Sh ble* gene from *Streptoalloteichus hindustanus*. This gene is small (375 bp) and confers resistance to the drug Zeocin in *E. coli*, yeasts (including *P. pastoris*) and other eukaryotes. Because the *ble* gene serves as the selectable marker for both *E. coli* and *P. pastoris*, the Zeo^{R} vectors are much smaller (~3 kb) and easier to manipulate than other *P. pastoris* expression vectors.

Vector diagram of pPICZa



3.1.10 *Pichia pastoris* culture

The wild type *Pichia pastoris* X33 strain was used. This strain was obtained from Invitrogen. *Pichia pastoris* cells were taken directly from a glycerol stock stored at -80° C onto solid YPD agar medium and incubated at 28° C. The Petri dishes were then stored at 4° C. Every two weeks, the yeast colonies were renewed, as they continue to grow at 4° C. From these YPD agar cultures liquid cultures can be prepared. The *Pichia pastoris* strains were cultured in liquid YPD medium for 20 hours at 28° C with shaking. The antibiotic Zeocin ™ (Invitrogen) was used for selection with a final concentration of 25 µg/ml.

YPD medium (Yeast Extract Peptone Dextrose) for 1 liter

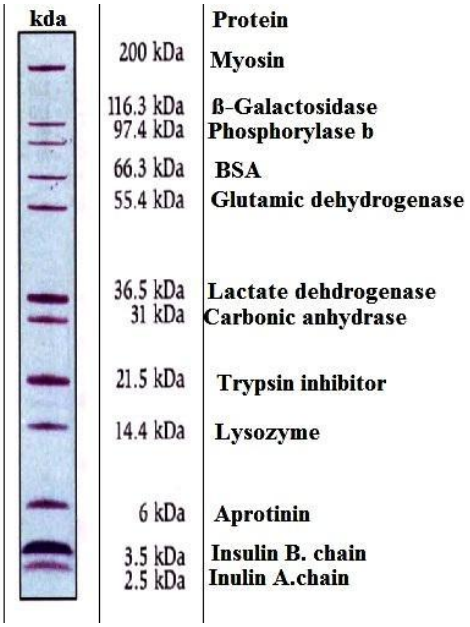
10g of yeast extract

20g peptone from casein

20 g glucose

3.1.11 Protein Marker

„Mark 12™ Unstained Standard“ Invitrogen



3.1.12 Software

CDPro (Sreerama and Woody, 2000)

3.2 Methods

3.2.1 Biochemical methods

3.2.1.1 Expression of recombinant Protein in *Pichia pastoris*

The yeast *Pichia pastoris* is a well-suited system of yeast species used for the production of recombinant proteins. As an eukaryotic organism, *Pichia pastoris* has many advantages of higher eukaryotic expression systems, especially post-translational modifications such as protein processing, protein folding and protein secretion into the medium, among which the later facilitates purification.

The *Pichia pastoris* expression system represents a very useful, fast, and cost-effective experimental tool for use in protein engineering investigations because it includes tightly regulated and efficient promoters and a strong tendency for respiratory growth as opposed to fermentative growth (Romanos *et al.,* 1992; Cereghino and Cregg 2000; Cregg *et al.,* 2009).

Compared with bacterial expression systems, the expression in *Pichia pastoris* has one great advantage: the yeast is able to carry out post-translational modifications, proteins fold properly and they can further be correctly O-and N-specific glycosylated (Higgins and Cregg 1998). A great variety of expression vectors for *Pichia pastoris* enable the facile development of multiple clonal linages containing the recombinant gene.

Using a yeast shuttle vector one can select clones that can express the protein of interest. In this research, the X33 wild type is used as host microorganism for the vector pPICZαA.

In this study, the vector pPICZαA induces extracellular protein secretion using the alpha mating factor (α-MF) secretion signal. This vector uses zeocin as antibiotic marker and allows plasmid integration to the AOX1 locus. These characteristics make this vector an optimal option when extracellular expression is required (Siren *et al.,* 2006). The wild type strain, X-33, is a positive methanol utilization strain. *E. coli* / *Pichia pastoris* shuttle vectors contain an origin of replication for plasmid maintenance. The *Pichia pastoris* system has the potential to express correctly folded proteins by using methanol as the sole carbon source (Higgins and Cregg 1998b), and produce large amounts of protein using the alcohol oxidase (AOX) promoter that drives the expression of the gene for the desired protein (Daly *et al.*, 2005).

The AOX enzyme allows the usage of methanol as the sole carbon source for the production of biomass and energy metabolism. There are two genes in *Pichia pastoris* that code for alcohol oxidase-AOX I and AOX II. The AOX I gene is responsible for the vast majority of AOX activity in the cell and expression of the AOX I gene is tightly regulated and induced by methanol to very high levels. AOX is undetectable in cells cultured in carbon sources such as glucose, glycerol or ethanol, but constitutes up to 30% of total soluble protein in methanol-grown cell, (Higgins, Busser *et al.*, 1998, Cereghino *et al.,* 2007).

I have used the *Pichia* X33 wild type strain to express our target protein. The vector pPICZαA was used to transform the target gene by the EasyComp method from Invitrogen. Initially, zeocin resistant transformants were selected on YPDS plates at 28-30° C for 4 days. To start protein expression, a single colony was grown on Buffered Minimal Glycerol medium (BMGH) with zeocin (25 µg/ml) at 28-30° C in the dark, on a rotary shaker at 180 rpm. The recommended cells for methanol induction should be in the OD range of 2-6 after 16-18 hours. The cells were harvested by centrifugation at 1500 xg for 5 min at room temperature (20 ºC). The cells were then grown on BMMH minimal medium (with methanol) for 2-5 days at 28-30°C in the dark on a rotary shaker at 180 rpm to achieve maximum protein expression. Methanol was added to a final concentration of 0.5 % (v/v).The fed-batch

phase is mainly characterized for growth under glycerol grow-limiting conditions. During this phase sub-products produced in the batch phase like ethanol are used by the yeasts, making them ready for the use of methanol (Zhang *et al.,* 2000).

The methanol induction phase in which the carbon source is fed at a growth limiting rate to avoid any prejudicial accumulation that may affect the cell. The induction phase activates the expression of the protein of interest (Cereghino *et al.,* 2000).

At each time points (hours): 0, 24 (1 day), 48 (2 days), 72 (3 days), 96 (4 days),1 ml of the expressed cultures were transferred to eppendorf tubes, centrifuged for 2-3 minutes at 14,000 rpm and samples were analyzed by SDS-PAGE.

BMGY (Buffered Glycerol-Minimal Medium) /preculture

700 ml	Autoclaved ddH_2O
100 ml	1 M potassium phosphate buffer pH 6
100 ml	10X YNB (13.4% Yeast Nitrogen Base without amino acids)
2 ml	500X Biotin (0.02% Biotin)
100 ml	10X GY (10% Glycerol)

BMMY (Buffered Methanol-Minimal Medium)/Induction

795 ml	Autoclaved ddH_2O
100 ml	1M potassium phosphate buffer pH 6
5 ml	10X Methanol (5% (v/v) Methanol)
100 ml	10X YNB (13.4% yeast nitrogen base without amino acids)
2 ml	500X Biotin (0.02% Biotin)

1L 1M potassium phosphate buffer pH 6: Combine 132 ml of 1M K_2HPO_4 (174,17 g/l) and 868 ml 1M KH_2PO_4 (136,1 g/l) and confirm that the pH = 6.0 (if the pH needs to be adjusted, use phosphoric acid or KOH) .

Methanol, Biotin, Yeast Nitrogen Base solutions were sterilized by filtration. All the other media were sterilized by autoclaving at 121°C for 20 minutes.

3.2.1.2 Centrifugation and storage

The culture medium with the recombinant protein was harvested by centrifugation at 13,000 rpm, 4 °C, for 30 min. The supernatant was collected and stored at 4°C and used for further protein purifications.

3.2.1.3 Purification of BN26 by reverse-phase chromatography using Amberlite XAD7

I have used the method of (Buhot *et al.*, 2004) with some modifications. Amberlite XAD7 is an industrial organic adsorbents matrix for the purification of peptides, proteins and small molecules from aqueous solutions by reversed-phase chromatography. The nonpolar Amberlite XAD resins are generally used for adsorption of organic substances from aqueous systems and polar solvent. Amberlite XAD7 is a polymeric adsorbent of white insoluble beads. It is a nonionic, aliphatic acrylic polymer which receives its adsorptive properties from its macroreticular structure with a large surface and the aliphatic character of its surface. This structure also provides the macroreticular Amberlite XAD7 with excellent physical and thermal stability. The separation mechanism in reversed phase chromatography depends on the hydrophobic binding interaction between the solute molecule in the mobile phase and the immobilized hydrophobic ligand, i.e. the stationary phase. As solute binds to the immobilized hydrophobic ligand, the hydrophobic area exposed to the solvent is minimized. In summary, separations in reversed phase chromatography depend on the reversible adsorption/desorption of solute molecules with varying degrees of hydrophobicity to a hydrophobic stationary phase.

The relatively non-polar media (Amberlite XAD7) can be used as stationary phase in this chromatography. As a mobile phase, a hydrophilic organic solvent in an

aqueous solution (such as acetonitrile, methanol and isopropanol) is used. A rapid separation and good resolution of individual components can be enabled by this chromatography. The more hydrophilic a substance is, the easier it is to move in the hydrophilic mobile phase and the faster it is eluted from the stationary medium. Conversely, the more hydrophobic a component in the sample, the more likely it will bind to the hydrophobic ligand in the stationary phase and be retained on the column. The Amberlite matrix was in a small column (height 10 cm, diameter 1 cm). The column was connected between a peristaltic pump and a UV detector. After 5 days of BN26 induction and recovering of the medium by centrifugation, the pH of the solution was adjusted to pH 2 with TFA (trifluoroacetic acid). After equilibrating the column with Amberlite XAD7 with 0.1% aqueous TFA, the culture supernatant was applied to the column and eluted with 0.1% TFA and 40% CH_3CN, 0.1% TFA. The proteins eluted with acetonitrile were neutralized with Tris base to pH 8 and the protein was concentrated and dried in the Speed-Vac for 5-7 hours or overnight to evaporate the acetonitrile. The dried protein fractions were resuspended in a buffer (the buffer is dependent on the experiment), desalted and prepared for the next experiments. The dried proteins can be stored for up to 4 months in the refrigerator.

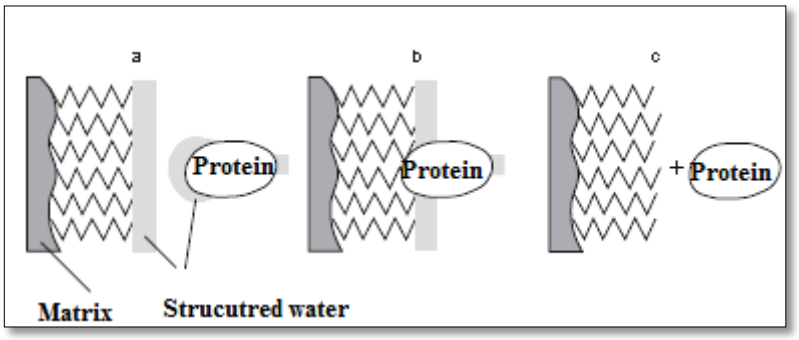


Figure 7: Interaction of a solute with a typical reversed phase medium from (Proteomics in practice book).

3.2.1.4 Protein desalting

Desalting of proteins was done using NAP5 columns from GE Healthcare (a SephadexTM G-25 grade column) with a bed volume of 0.5 ml.

Apply 500 µl protein sample with salt on NAP-5 column

↓

200 µl elute and discard elute (containing no protein yet

↓

Elute 300 µl and collect (1st eluation)

↓

Then 500 µl eluate again on new freshly equilibrated NAP-5 column

↓

Elute 700 µl and collect eluate is then contain no salt. The total elution volume is then 1000 µl

Figure 8**:** Schematic representation of the desalting method using Nap-5 columns.

The eluted free-salt protein solution was used for further experiments.

3.2.1.5 Protein precipitation

3.2.1.5.1 Ammonium sulfate precipitation

To precipitate the isolated protein, the solution was saturated with 60% ammonium sulfate (390 g for 1L) and stirred for one hour in the refrigerator. The precipitated protein solution can be stored for several months at 4°C.

3.2.1.5.2 Acetoneprecipitation

Four volumes of ice-cold acetone was mixed with one volume of the protein sample and incubated at least one hour at -20 °C. Then, the precipitated proteins were removed by centrifugation for 30 min 15.000 g, 4 °C. The sediment was washed with 90% acetone, centrifuged again, and dried at room temperature or at 37 °C. For further use the pellet was resolved in the desired buffer.

3.2.1.6 Colorimetric determination of protein concentration by Bradford test

In the Bradford test the basic and aromatic amino acid side chains of proteins form a protein-dye complex with Coomassie Brilliant Blue G250 that appears blue in solution. The absorption maximum of this complex is 595 nm. Due to the increase of the absorbance at OD 595 in comparison with a calibration curve, the concentration of a protein can be determined. The intensity of the colour is proportional to the amount of the protein in the reaction mixture (Bradford 1976).

100 µl protein samples were mixed with 700 µl H_2O and 200 µl of the Coomassie solution (Roti-Quant) solution. After 5 to 30 minutes of incubation at room temperature, the samples were measured spectrophotometrically at 595 nm. For reference 800 µl H_2O and 200 µl Coomassie solution (Roti-Quant) were used. A calibration curve was created with (BSA) bovine serum albumin prepared with concentrations 5-2000 µg / ml. The determination of the protein concentration was carried out by comparison with the values of the calibration curve.

3.2.1.7 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Tricine-SDS-PAGE is commonly used to separate proteins in the mass range 1-100 kDa. It is the preferred electrophoretic system for the resolution of proteins smaller than 30 kDa. SDS-PAGE was carried out using a Tris-Tricine discontinuous system as outlined by Schägger and von Jagow (1987). SDS-Gel electrophoresis is a biochemical method used to separate proteins according to their molecular weight. In order to achieve separation of proteins according to their size, denaturation by SDS and heat is necessary to eliminate the influence of protein folding and structure.

ß-mercaptoethanol is added to cleave disulfide bonds. Polymerization is catalyzed by addition of TEMED and APS. Gels are poured according to the method of Schägger and Jagow to achieve better separation of low-molecular mass proteins. Samples were mixed with SDS sample buffer and incubated at 95° C for 5 min. In each lane 20-25 µl sample were applied. The gels were run for 30 min at 30 V continuing to 150 V for 2-3 h. The gels were then stained with Coomassie staining solution by shaking for a minimum of 1 h. Distaining was performed overnight until the background was clear. By comparison with a standard protein mixture (Mark 12
™ Unstained Standard, Invitrogen) an estimate of the size of proteins to be analyzed is possible.

Table 9 Gel-, Cathode- and Anode buffer

	Gelbuffer	**Cathode buffer**	**Anode buffer**
SDS	0.3 %	0.1 %	
Tris	100 mM	200 mM	50 mM
Tricin		100 mM	
pH	8.4	8.25	8.9

Sample buffer (3X)

0.135 M Tris-HCl pH 6,8

30% Glycerin

0.03% Bromophenol blue

0.15 M (Dithiothreitol) DTT

3% SDS (Sodium dodecylsulfate)

3.2.1.7.1 Solutions used

Acrylamide solution: mixed solution of 30% acrylamide: 0.8% N, N'-methylenebisacrylamide, 5:1

APS: Ammonium persulfate, 10% (w/v) solution in distilled water.

TEMED: N, N, N ', N'-tetramethylethylenediamine

3.2.1.7.2 Coomassie-Staining and –Destaining

Table 10: Coomassie-staining and -destaining

Staining	Destaining
45 % Methanol	45 % Methanol
10 % Acetic acid	5 % Acetic acid
0.2 % Coomassie Brillant R-250	

Table 11: Chemicals for one gel

Chemicals	1x Seprating gel (16%)	1x Separting gel (10%)	1x stacking gel
Acrylamide 30 %+ Bis acrylamide 0,8%	3,09 ml	0,83 ml	0,4 ml
Gel buffer	1,87ml	0,83 ml	0,78 ml
Glycerin	0,62 ml	----	-----
H_2O	0,03 ml	0,84 ml	1,925 ml
TEMED	3.125 µl	4 µl	11 µl
AMPS 10 % (ammoniumpersulphate)	25 µl	15 µl	37,5 µl

3.2.1.7.3 Drying and Storage of SDS-polyacrylamide gels

To preserve the gels, they were incubated in 5% (v / v) glycerol for 5 min and dried directly between two cellophane sheets.

3.2.1.8 Western blot

3.2.1.8.1 Protein transfer to a nitrocellulose membrane

Western blotting allows the detection of specific proteins. After an electrophoretic separation, non-stained SDS gels were blotted on a nitrocellulose membrane on which proteins bind unspecifically due to hydrophobic and charge interactions and identified by specific antibodies. The transfer of electrophoretically separated proteins to nitrocellulose membranes in accordance with Towbin (Towbin *et al.,* 1979) is performed in the tank-blotting with the blot apparatus from BioRad (Germany).

SDS-gels were put in the transfer sandwich and equilibrated for a few minutes in transfer buffer. Then, the gels were packed in a bubble-free sandwich blot between two sponges with a nitrocellulose membrane, so that the nitrocellulose membranes (Protran ® nitrocellulose transfer membrane, Schleicher & Schuell, Germany) face the anode. The sandwich blot was inserted into a tank filled with transfer buffer (10x reservoir buffer = 190 mM Tris, 1.9 M glycine, 20% methanol). At 100 mA current, 14 hours and a temperature of 4°C, the transfer of the proteins from the gel to the membrane was carried out.

The blot is incubated with a generic protein (such as milk protein Casein) to bind to any remaining sticky places on the nitrocellulose. An antibody is then added to the solution which is able to bind to its specific protein. The antibody has an enzyme (e.g. alkaline phosphatase or horseradish peroxidase) or dye attached to it which cannot be seen at this time. The location of the antibody is revealed by incubating it with a colorless substrate that the attached enzyme converts to a colored product that can be seen and photographed.

Western blot buffer (Transfer Buffer)

0,025 M TRIS/pH= 8,3
0,192 M Glycin (w/v)
20% MeOH (v/v)

For His BN26 antibodies dilutions

Monoclonal Anti-polyHistidine, Clone HIS-1 (H1029) 1:3000
Anti-Mouse IgG-Alkaline Phosphatase 1:30000

3.2.1.8.2 Ponceau staining

After transfer of the protein to nitrocellulose membrane the Ponceau staining was used to check the transfer efficiency of the protein and to make it visible on the membrane.

Immediately after the transfer the membranes were dyed in the Ponceau staining solution 0.2% (w/v) Ponceau S red, 3% (v/v) trichloroacetic acid, 3% (w/v) sulfosalicylic acid for 10 minutes. Subsequently, they were washed twice with H_2O. When using the default marker, the marker bands are marked on the membrane.

Ponceau S-colouring solution
0,2 % Ponceau S Red (w/v)
3% TCA (v/v)
3% sulfosalicylic acid (w/v)

3.2.1.8.3 Blocking of the free surfaces of the membranes.

After transfer of the protein, the membrane was incubated 3 times for 15 min in 20 ml of blocking buffer with gentle shaking, to block the free binding sites on the membrane. MPT was used as a blocking buffer with casein. Then the membrane was incubated for 1 hour at room temperature or overnight at 4° C with the first antibody diluted 1:500 in MPT buffer (anti-WAX9). After washing 3 times for 5 minutes in 30 ml of wash buffer MPT, the membrane was incubated with the secondary antibody

(Goat anti-Rabbit IgG (H + L) HRP conjugate Bio-Rad diluted 1:3000 in MPT) for 30 minutes at room temperature followed by three times 5 minutes washes with MPT buffer.

Table 12: **MPT, Blocking and AP-buffers**

10x MPT-buffer	**AP-buffer (Alkaline Phosphatase)**	**Blocking buffer**
100 mM Tris	100 mM Tris	1x MPT
1.5 M NaCl	100 mM NaCl	3 g/l Casein
0.5 % Tween	50 mM MgCl2	
pH 7.4 with HCl	pH 9.5 with HCl	

3.2.1.12 Development: staining of the bound proteins

For staining the chromogenic substrate BCIP (5-Bromo-4-Chloro-3-Indolyl-Phosphate) NBT (Nitro blue tetrazolium chloride) was used. AP buffer (Alkaline phosphatase buffer) catalyzes the cleavage of the phosphate group of BCIP and converts it to the corresponding indoxyl. This is oxidized by NBT and dimerizes to the deep blue, insoluble coloured precipitate (Sambrook and Russell, 2001). After incubation with an AP-conjugated secondary antibody, the membrane was equilibrated for three times for 15 min at RT in the AP buffer. The reaction solution containing the substrates NBT and BCIP was left on the membranes until a colour (dark blue precipitate) was visible. The reaction was stopped by rinsing the membrane in water.

To keep the background low, the colour reaction was performed in the dark at room temperature for 5-30 minutes. After reaching the desired colour development, the reaction was terminated by washing in H_2O, and the membrane was dried in air. The staining was done without shaking.

Stock solutions

BCIP Stock (-Bromo-4-Chloro-3-Indolyl-Phosphate): 50 mg BCIP
1 mL 100% DMF (Dimethylformamide)
NBT Stock (Nitro blue tetrazolium chloride): 50 mg NBT
1 mL 70 % DMF (Dimethylformamide)

Reaction solution for one membrane
10 mL AP-Puffer
33 µL BCIP Stock
66 µL NBT Stock

3.2.1.9 Isolation of Thylakoids and Cryoptotectin protein

3.2.1.9.1 Isolation of thylakoid membranes from non-hardened spinach (Hincha and Schmitt, 1992)

Thylakoid membranes were isolated from non-hardened spinach leaves as follows: Thylakoids are sensitive to elevated temperatures. Therefore all solutions and glassware should be cooled to 0-4° C. Isolated membranes should always be kept on ice. All centrifugation steps take place at 4°C. 50 g of spinach leaves were homogenised in 100 ml of homogenization buffer in a blender (2 L mixer) for approximately 10 sec. The homogenate was filtered through a nylon mesh to remove coarse particles. The filtrate was centrifuged for 5 min at 6000 rpm and 4° C, the supernatant was discarded, and the pellet was resuspended in approximately 50 ml of washing solution (10 mM NaCl). This is most easily performed using a Pasteur pipette for 3 or 4 times. After centrifugation for 5 minutes at 8000 rpm and 4°C the supernatant was discarded. This washing procedure was repeated twice in washing solution (10 mM NaCl).

The thylakoid suspension was diluted with washing buffer to a concentration of at least 1mg/ml.

Thylakoid isolation buffer (1L):
240 mM Saccharose
160 mM NaCl
1 mM $MgCl_2$ (Hexahydrate)
1 mM $MnCl_2$ (Dihydrate)
2 mM EDTA
1 mM KH_2PO_4
50 mM Tris
pH 7.8 37 % HCl adjustment
Immediately before use fresh added: 1.25 mM Na Ascorbat und 3.3 mM Cystein.

3.2.1.9.2 Chlorophyllestimation (Arnon, 1949)

10 µl of the thylakoid suspension were mixed with 990 µl of 80% acetone in an Eppendorf tube and centrifuged for 2 minutes in benchtop centrifuge at 14000 rpm. The absorbance of the supernatant at 663 nm and at 645 nm was measured against 80% acetone as the reference in a glass cell.

Chlorophyll concentrations can be calculated by the following equation:

$$20{,}2 \times (A_{645}) + 8{,}02 \times (A_{663}) = X \text{ mg Chlorophyll /10 ml}$$

3.2.1.9.3 Isolation and purification of Cryoprotectin from *Brassica oleracea*

This method was used as in (Sieg *et al.*, 1996). Hardened-cabbage leaves (1kg) were homogenized in a 5-L laboratory blender in 600 ml of an ice-cold homogenization buffer. The homogenate was filtered through a 50 µm nylon mesh and then centrifuged for 30 min at 23,000 x g at 4°C. The supernatant was incubated at 100 °C in a boiling water bath for about 8 minutes. Then, the solution was immediately transferred to an ice-water bath and centrifuged at 23,000 g for 15 minutes. The supernatant was adjusted with acetic acid to a pH of 4.0, centrifuged at
23,000 g for 15 minutes and nylon 6 matrix is added to the supernatant until the solution became cloudy. Further precipitation was done by the addition of solid ammonium sulfate to 60% (390 g for 1L). The solution was then stirred for one hour at 4°C. The precipitated protein solution can be stored for several months at 4°C.

Homogenisation buffer:
50 mM Tris,
2 mM EDTA,
2 % Polyclar (w/v)
Freshly added: 300µM Mercaptobenzothiazole
pH 7.8 with 99 % Acetic acid.

3.2.1.10 Cryoprotective activity of BN26 (Hincha and Schmitt, 1992a)

Cryoprotection of spinach thylakoids was tested using isolated non-hardened spinach thylakoids. Isolated thylakoids have been used as a model membrane system to determine whether a cryoprotectant protein can protect membranes against freezing injury (Hincha *et al.*, 1989; Hincha and Schmitt, 1992).

When thylakoid membranes are damaged by freezing, the vesicles lose their contents and collapse, which can be measured as a decrease in the packed thylakoid volume. When thylakoids are frozen in a low osmolarity and then thawed, they damage. In this case they have less volume compared to a normal case. The overexpressed BN26 protein and the control samples were used to examine their cryoprotective activity compared to BSA. *Pichia pastoris* X33 activity was also tested.

The tested samples can be divided into three groups:
1-100 µl Thylakoid suspension + 100 µl protein sample (BN26)
100 µl Thylakoid suspension + 100 µl sample solution (zero control)
100 µl Thylakoid suspension + 100 µl CPP (positive control)

The tested additives were suspended in the sample solution (10 mM sucrose, 1 mM $MnCl_2$, 1 mM $CaCl_2$) and desalted over NAP5 columns. 100 µl of the protein samples were taken from a 1 mg/ml protein stock solution, mixed with 100 µl of thylakoid suspension (from 1 mg of chlorophyll/ml) and frozen at -20° C for 2-3 hours. The final protein concentration of the tested proteins was 0.5 mg/ml. After the incubation period the samples were thawed rapidly (within 2-3 min) in a water bath at

room temperature. For the volumetric assay, the samples were diluted with an equal volume of 10 mM $MgCl_2$ and the packed thylakoid volume was measured after hematocrit centrifugation for 15 min at 12,000 rpm. Upon adding $MgCl_2$, thylakoids stack together to form grana that pack more compactly than the NaCl-washed, unstacked thylakoids. The measurements were taken from each sample and averaged. Upon adding $MgCl_2$, thylakoids stack together to form grana that pack more compactly than the NaCl-washed, unstacked thylakoids.

Therefore, the volume of sedimented thylakoids shows a signal for the quantification of the effect of cryoprotective proteins. For calibrating a volume with the same amount of cold-sensitive thylakoids without freeze-thaw cycle is determined, which represents the fully protected thylakoids. Protection was calculated relative to control samples that were incubated for the same time at -20ºC (0% protection) or 0 ºC (100% protection) in the absence of added protein (Fig. 9).

The 100% frost-protected thylakoid volume (100% cryoprotective activity) is given by the difference between the 0° C and the -20° C control. The cryoprotective activity was calculated using the following formula:

TKV (+ P, -20 ° C) - TKV (-P, -20 ° C) / TKV (-P, 0 ° C) - TKV (-P, -20 ° C) x 100 = % cryoprotective activity.

TKV: Thylakoid pellet height (mm)

+P: with protein sample

-P: control sample without protein sample

Table 13: Washing, sample and test solutions

Sample solution	Washing solution	Test solution
10 mM Saccharose	10 or 5 mM NaCl	10 mM $MgCl_2$
1 mM $MnCl_2$		
1 mM $CaCl_2$		

The sample solution contained manganese and calcium which also promote stacking, because the cryoprotective property of the protein is cation-dependent. It needs both calcium and manganese (Sieg *et al.,* 1996), and this property is similar to some lectins from legumes, which are also calcium and manganese-dependent.

BN26, CPP, BSA in different concentrations were also mixed with 100 µl thylakoidsuspension (1mg/ml) and tested for cryoprotective activity.

The previous test was repeated in the presence of different protein combinations in order to investigate if different cryoprotectants are more efficient if combined together.

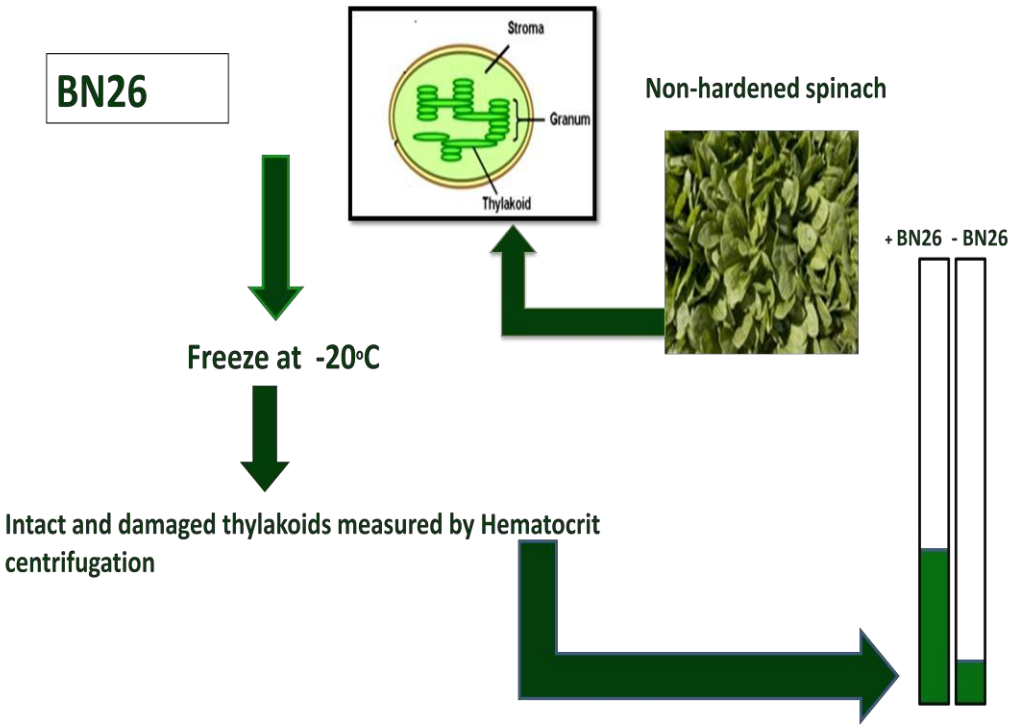


Figure 9: Schematic illustration of the cryoprotective assay.

3.2.1.11 Binding activity of BN26 with Thylakoid Membranes

Thylakoids were mixed with the test proteins, incubated at 0°C for 15 min and then subjected to three rounds of centrifugation (5min at 14,000 xg). Aliquots from the supernatant and the pellet were used for protein detection by SDS-PAGE and western-blot analysis. 1 volume of each aliquot was added to 4 volumes 100% acetone and incubated for at least 1 h at -20 °C to remove the chlorophyll from the SDS By SDS gel electrophoresis and Western blot, the samples were tested. When the proteins bind to the membrane, they will remain after centrifugation in the pellet samples. Non- bound protein samples to the thylakoid membranes remain in the supernatant.

When the proteins bind to the membrane, they will remain after centrifugation in the pellet samples. Proteins, which didn't bind to the thylakoid membranes, remain in the supernatant. This test gives us a clear signal if the proteins bind to the thylakoid membranes or not. By SDS gel electrophoresis and western blot, the samples were tested.

Pseudomonas syringae allantoicase (ALC) was used as a negative control and CPP Cryoprotectin was used as a positive control.

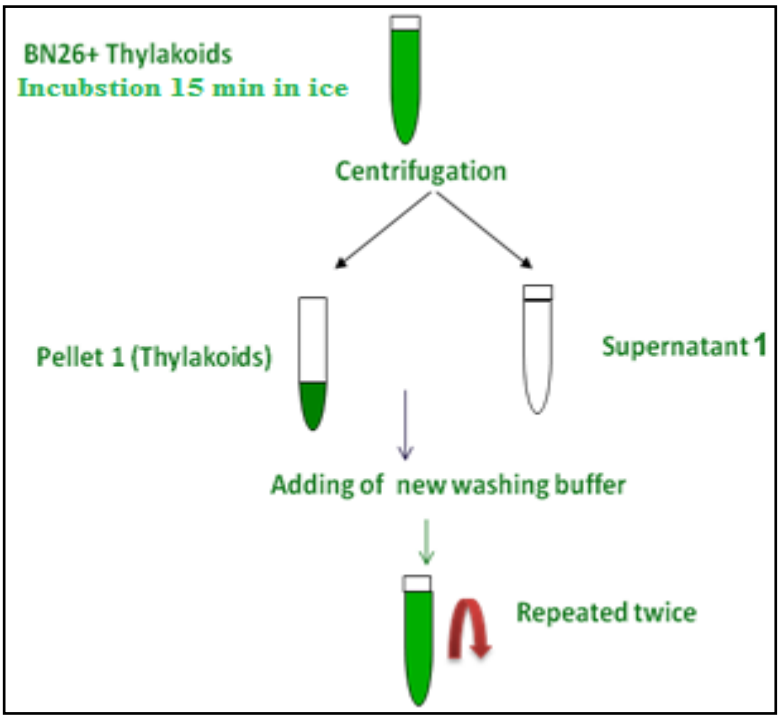


Figure 10: Shematic rrepresentation of the binding assay to thylakoid membranes.

3.2.1.12 Protease Sensitivity Assay

75 µl (100 µg) of BN26 and lysozyme in the tested buffer was added to 5 µl Trypsin (100 ng) at 37° C for 1 hour. The total reaction volume was 150 µl. Samples (5 µl) were taken in time intervals from 0-60 min and the reaction stopped by adding 15 µl loading buffer and heating at 95 °C for 5 minutes. Then the samples were analyzed on a 10–20% acrylamide gradient gel. Digestion after freezing to denature Lysozyme and freeze-stressed BN26 was also tested by incubation with 100 ng of trypsin for 1h at 37°C.Then 2 aliquots were removed and the reaction was stopped by the addition of SDS-PAGE loading buffer and immediate boiling for 5 min and analyzed by SDS-PAGE by running on a 10-20% polyacrylamide gradient gel.

Buffer

50 mM Tris-HCl pH 7,5
150 mM KCl
10 mM$CaCl_2$

3.2.1.13 Refolding (Chaperone) activity assay of BN26

I have examined the refolding activity of BN26 using firefly luciferase as a substrate (Lee and Vierling, 2000). 1 µM firefly luciferase (Promega) (0.061 mg/ml^{-1}) was diluted (1:40) approximately in either 1 µM (0.087 mg/ml) BN26 or 1µM (0.097 mg/ml) COR15A or 1 µm (0.066 mg/ml) BSA or alone and completely denatured in 25 mm 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), 5 mM $MgCl_2$, 150 mM KCl, and 2 mM dithiothreitol, pH 7.5 (denaturation buffer) for 10 min at 42 °C. Then an equal concentration of BN26 or CORA or BSA solution was further added into the denatured enzyme mixtures, and the refolding reaction was started at 30° C in presence and absence of 2.5 mM ATP. The reactions were done in siliconized tubes. Luciferase activities were determined by adding 50 µL of the refolding reaction to 100 µL of Luciferase Assay Mix (Promega) and monitoring light emission in the

luminometer. Luciferase activities are expressed as percentages relative to that of luciferase activity in BSA prior to heat denaturation which was defined as 100%.

3.2.1.14 *In vitro* assays to measure lactate dehydrogenase stabilization under different stresses

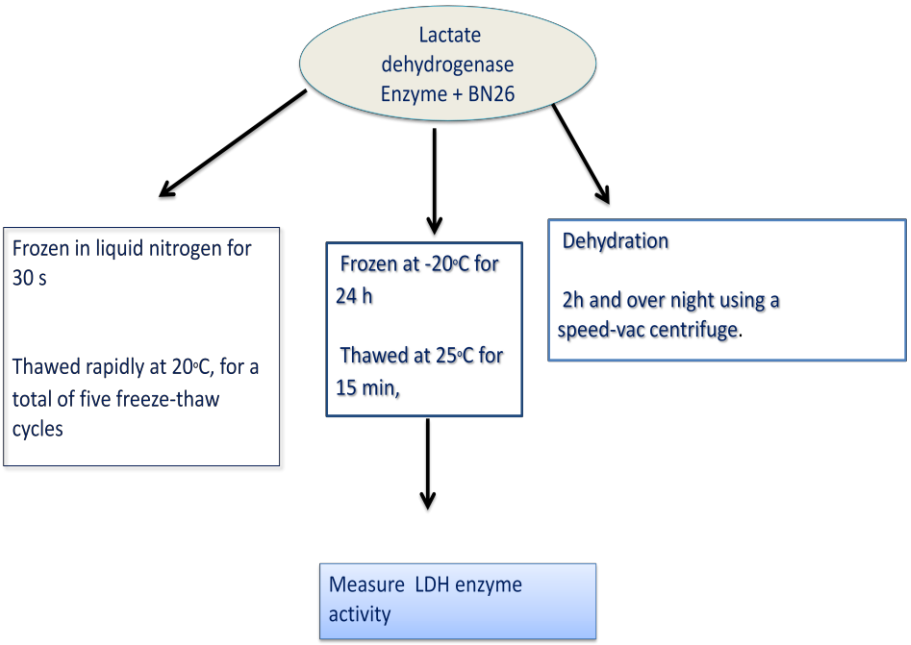
Lactate dehydrogenase is an enzyme that is very sensitive to both freezing and drying. It is a convenient model to study protein stabilization. The LDH EC# 1.1.1.27. Rabbit muscle lactate dehydrogenase-5 assay was carried out as described by Tamiya *et al.* (1985).

For freezing experiments

1.5 µl of 5 µM BN26 and other tested proteins except for Sucrose 50 µg, BSA 50 µg in 10 mM K_2HPO_4, pH 7.5 was distributed on a 96-well plate in 3 replicas. 5 plates were prepared and 1.5 µM LDH from rabbit muscle (Roche) in 10 mM K_2HPO_4, pH 7.5, in a concentration 0.5µM (Tetramer) were added per well. LDH in one plate was measured immediately after adding 200 µl of LDH sample buffer containing 80 mM Tris-HCl pH 7.5,100 mM KCl, 2 mM pyruvic acid (Sigma), 0.3 mM NADH (Sigma).

For multiple freeze-thaw cycles, 4 other plates were prepared and sealed well before frozen in liquid nitrogen for 2 minutes for one to four times, thawed at RT in water bath in-between and measured directly. Unfrozen LDH samples were assayed for enzyme activity at the time of addition as well as the next day to establish prefreeze–thaw activity (100%). Relative LDH activity (%) resulting from cryoprotection by heat stable and standards was determined by calculating protein activity of frozen LDH at each concentration as a percentage of prefreeze LDH activity (Fig. 11).

For drying experiments: LDH solutions in the presence and absence of proteins were either 70% or 100% dehydrated using a speed-vac centrifuge. The samples were then rehydrated to the original volume with water, and LDH activity was measured.



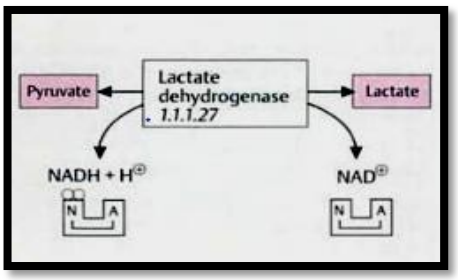


Figure 11**:** Schematic representation of an *in vitro* assay of LDH

3.2.1.15 Pull-down assay

Pull down assays were performed on micro columns packed with 0.25 ml Ni-Sepharose 6 Fast Flow resin (GE Healthcare). The proteins were washed five times with 1 ml binding buffer and eluted from the column with 300 µl elution buffer each (20 mM HEPES, pH 7.5, 50 mM NaCl, 200 mM imidazole, 0.005% Triton X-100). This assay was used to test the association of BN26 and LDH. Fig. 12 shows the idea of the pull-down assay.

The protein and lactate dehyrogenase were suspended in 1 ml 10 mM potassium phosphate buffer pH 7.5 and desalted and 4 columns were used in parallel:

BN26 prefrozen + LDH unfrozen and applied directly after thawing.

BN26 unfrozen and applied directly after thawing + LDH unfrozen.

P.s ALC (300 µg/ml) (–ve his-tagged) control protein frozen + LDH unfrozen and applied directly after thawing.

LDH alone unfrozen

The proteins were washed 5 times with 1 ml binding buffer (20 mM HEPES, pH7,5, 50 mM NaCl, 20 mM imidazole, 0,005% Triton X-100), eluted from the column with 500 µl elution buffer (500 mM imidazole) and the fractions were pooled

BN26 was 300 µg/ml= 25 µM

LDH was 700 µg/ml= 5 µM

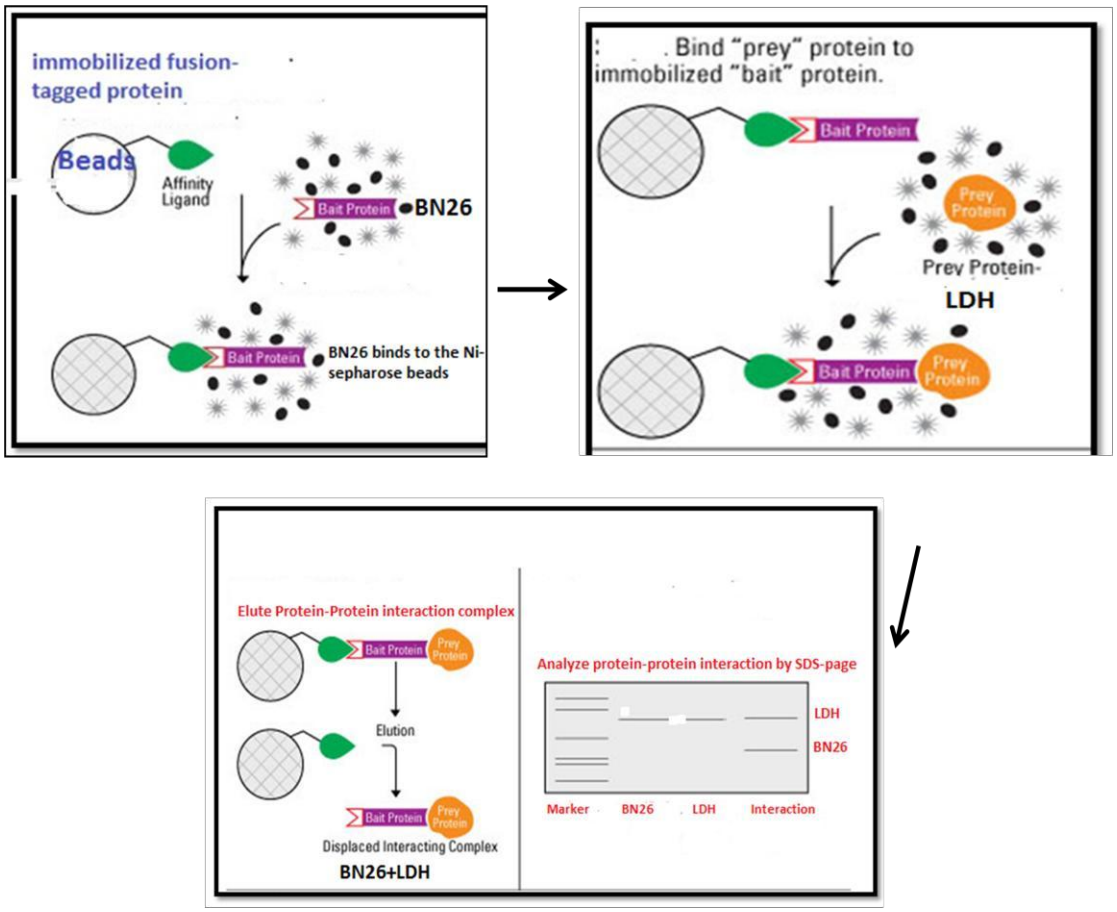


Figure 12: Schematic representation of the Pull-down assay

3.2.2 Biophysical Methods

3.2.2.1 Circular dichroism spectroscopy (CD)

Far-UV circular dichroism (CD) spectroscopy is a technique used for analyzing the structure of optically active substances like proteins.

If circularly polarized light is directed through an optically active substance, it shows an unequal absorbance of left-hand and right-hand circularly polarized light. Circular dichroism is defined as the difference in the absorption of left and right-handed circularly polarized light by a sample. It detects the amount (or lack) of secondary structure. The common secondary structure elements of proteins (α- helix, β-sheet and random coil) exhibit distinctive CD-spectra in the far-ultraviolet region (190-250 nm) where the peptide bond serves as chromophore. By using a variety of computer algorithms, the approximate fraction of each structure type present in a protein can be determined.

CD spectra were obtained with a Jasco-715 spectropolarimeter (Jasco Instruments) at the Max Planck Institute for Molecular Plant Physiology Golm, in cooperation with Anja Thalhammer (PhD student, AG Hincha).

Proteins were measured in the dry and hydrated state. For the measurement of dry samples, 50 µl of 2 mg/ml protein dissolved in distilled water were spread on a CaF_2 window and dried in a desiccator over a fresh silica gel pad over night at 28 ºC. Samples were put in a spectropolarimeter which was continuously purged with N_2 to remove perturbing oxygen. To minimize the oxygen in the measuring cells, samples were left in the cell for 5 minutes before the measurement was started. Subsequently four CD spectra were recorded per sample (1 nm data pitch, 4 s gauging per data point, 1 nm band width). After cleaning the CaF_2 windows, they were measured once again for background subtraction.

Hydrated samples were measured in an approximate concentration of 0.75 mg/ml in D_2O in 0.1 mm Suprasil cuvettes under the same conditions as the dry samples. Background subtraction was done by filling the cuvette with D_2O. As quantification of proteins by standard colorimetric methods is unreliable because of the highly biased amino acid composition, we used the absorption at 193 nm measured in parallel with the CD signal.

For data analysis, the background was subtracted and data were converted from machine units to molar ellipticity unit s ($\Delta\varepsilon$) using the following formula:

$$\Delta\varepsilon = \frac{0.1 \times \text{machine unit sample} \times \text{MMW}}{d \times OD_{193} \times 3298}$$

d = cuvette width [cm]
MMW = mean molecular weight (MW of protein / number of amino acids)

As protein concentration is an important parameter for normalization of the spectra, the approximate protein concentration via a BSA standard curve was not sufficient for this purpose. For a more reliable determination, the logarithmic correlation between absorption maximum at 193 nm and protein concentration was used.

Data were analyzed by means of the open source software CDPro, using the algorithms CONTINLL, CDSSTR and SELCON 3 with the reference settings 6 and 7 which both contain a certain number of denatured proteins. The inclusion of denatured reference proteins was important for data analysis because the hydrated BN26 proteins are largely unstructured and a good estimation of the secondary structure can only be performed when the dominating structural element is present in the reference set.

3.2.2.2 Liposome formation

Liposomes are vesicular structures consisting of hydrated polar lipid bilayers. The chloroform-dissolved lipids as shown in (Table 8) were mixed in appropriate ratios (10 mg) in a glass reaction tube and then dried under a stream of N_2 on a 60ºC thermoblock and stored under vacuum overnight in the speed-vac to remove traces of chloroform.

Table 8: List of different lipids used in liposome leakage experiment

Lipid	Abbreviation	Proportion expressed as % (w/w)
Egg phospatidylcholine	EPG	15%
Monogalactosyldiacylglycerol	MGDG	40%
Sulfoquinovosyldiacylglycerol	SQDG	15%
Diagalactosyldiacylglycerol	DGDG	30%

3.2.2.3 Effect of Freezing on liposome leakage (Hincha *et al.*, 1998; Hincha *et al.*, 2002)

Liposome leakage normally occurs with the removal of water for example during drying, freezing or osmotic stress. When the dye carboxyluorescein (CF) is enclosed into liposomes, but not in the surrounding medium, leakage can be measured as the ratio of fluorescence in the samples before and after the addition of the detergent TritonX-100, which disrupts the liposome membranes and leads to complete leakage. The CF fluorescence dye has self-quenching properties so when the dye is trapped inside the liposomes at high concentrations no fluorescence can be measured and fluorescence is increased when the dye is released into the medium. 10 mg in total of lipids were prepared and rehydrated in 250 µl of 100 mM CF, 10 mM TES and 0.1 mM EDTA (pH 7.4) and extruded (15 times) using a Liposofast hand-held extruder (Avestin, Ottawa, Canada) with 100 nm pore polycarbonate membrane filters (Fig. 13).

Then the sample was purified over a Nap-5 column to remove non-encapsulated CF. Therefore, about 0.5 ml of the first coloured fraction were collected and filled up with TEN-buffer (10 mM TES and 0.1 mM EDTA, pH 7.4) to reach a final liposome concentration of 10 mg/ml. 20 µl of liposomes were mixed with BN26 or other control proteins in a (v/v) ratio to reach a final liposome concentration of 5 mg/ml. Final protein concentrations were 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml to reach protein: liposome ratios of approximately 1:6, 1:12, and 1:25, respectively. 12 µl of protein-liposme mixture were pipetted into the wells of a 96-well PCR-plate in three replica, including samples without protein as a control. The plate was sealed and frozen in an ethylene glycol bath to -20ºC for 2h. Controls were incubated on ice for 2 h. After 15 min, potentially non-frozen samples were touched with a spatula frozen in liquid nitrogen to induce sample freezing. After 3h of incubation, samples were warmed quickly to room temperature in a water bath. After thawing, Samples were transferred to a black 96-well microtitter plate, a 6 µl reference of unfrozen liposomes was added, and samples were diluted in 300 µl TEN-buffer and mixed by pipetting.

Fluorescence was determined in a Fluoroscan Ascent fluorescence plate reader (excitation wavelength 444 nm, emission wavelength 555 nm). To completely disrupt the liposomes 5 µl of 1% Triton X-100 were added subsequently. After mixing, fluorescence was determined again to identify maximal fluorescence. Liposome leakage was calculated as percentage of maximal fluorescence. In experiments where leakage values above 100% were obtained, values were normalized to the maximal value.

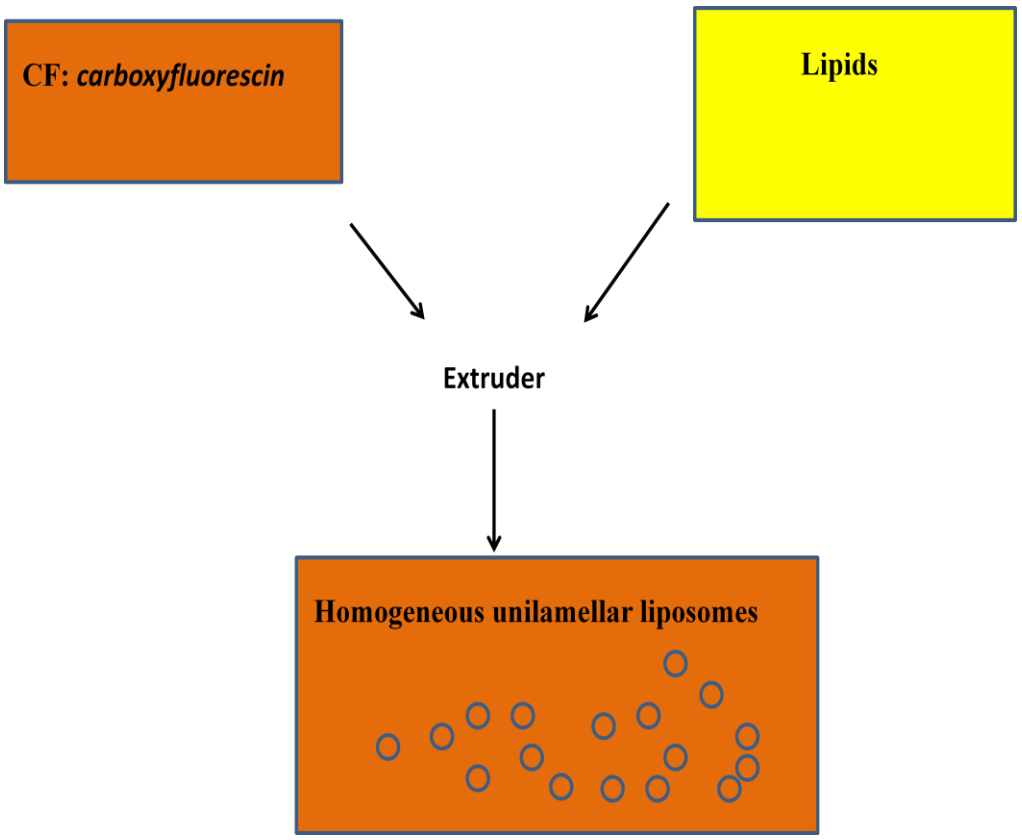


Figure 13: Schematic representation of liposome extraction

Statistical analysis

Significant results were determined using the independent unpaired two-tailed Student's t-test with the values obtained by BSA as reference if not mentioned otherwise.

Significant values were defined as

$0.05 > p > 0.01$	significant (∗)
$0.01 > p > 0.001$	very significant (∗∗)
$p > 0.001$	highly significant (∗∗∗)

4.1 Expression of recombinant BN26 protein in *Pichia pastoris*

Pichia pastoris (yeast) was used as a cellular host for the expression of BN26. *Pichia pastoris* is an eukaryote, and therefore provides the potential for producing soluble, correctly folded recombinant proteins that have undergone all the post-translational modifications (Daly and Hearn, 2005). Selection of positive yeast transformants via Zeocin resistance and induction of protein expression using a methanol inducible system were performed as described in experimental procedure. In order to establish the expression system for BN26, samples at different time points (every 24 hours up to 96 hours) of the induction period were taken. Since we used a vector with alpha factor secretion (pPICZαA), my target protein should be secreted in the supernatant. The vector pPICZαA contains the methanol-inducible alcohol oxidase promoter (P (AOX1)) to express the fusion genes (De Schutter *et al.*, 2009). The supernatant was separated from the yeast cells by centrifugation and analyzed by SDS-PAGE followed by Coomassie staining and western blot analysis (Fig. 14). To enable detection via antibodies a His-tagged version of BN26 was constructed. The expressed BN26 was without transit peptide.

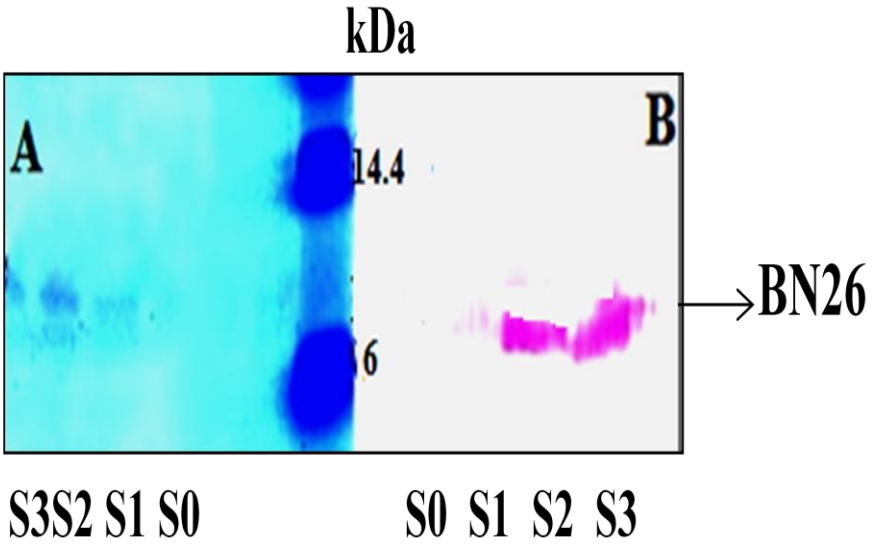


Figure 14: Expressed His-BN26 in *Pichia pastoris*. At 24 hours intervals, samples were taken from the supernatant removed from the yeast cells by centrifugation (S0= 24 hours (sample from BMGH medium flasks); S1= 48 hours; S2= 72 hours; S3= 96 hours (samples from BMMH medium flasks). Supernatant aliquots were then separated via SDS-Page and analyzed by Coomassie staining (A) and Western blot (Anti-His) (B). The samples consist of 50 µl protein sample and 50 µl sample buffer together, of which 20 µl per gel lane was applied. The proteins were separated for 1.5 hours at 150 volts.

As shown in Fig. 14 the induced protein can be readily detected in either Coomassie stained protein gels or on Western blots. The bands become stronger at the end of the induction period at time point S3 (96 hours). The detected protein shows a clear band between 6-14 kDa. This is in good accordance with the calculated molecular weight of BN26 of 8.7 kDa. BN26 could be successfully expressed as a recombinant protein in *Pichia pastoris.*

4.2 Purification of BN26 by reverse-phase chromatography

At the end of the induction period (4 days), the medium was recovered after centrifugation. The pH of the supernatant was adjusted to 2 by the addition of trifluroacetic acid (TFA). Then the supernatant was loaded onto Amberlite XAD7 preequilibrated with 0.1% aqueous TFA (Buhot *et al.*, 2004). After washing with 100 ml 0.1% aqueous TFA and 10% CH_3CN, 0.1% TFA, the supernatant was eluted with 50 ml of 40% CH_3CN, 0.1% TFA. Before CH_3CN evaporation under vacuum, the eluted fractions were adjusted to pH 8.0 with 1 M Tris.

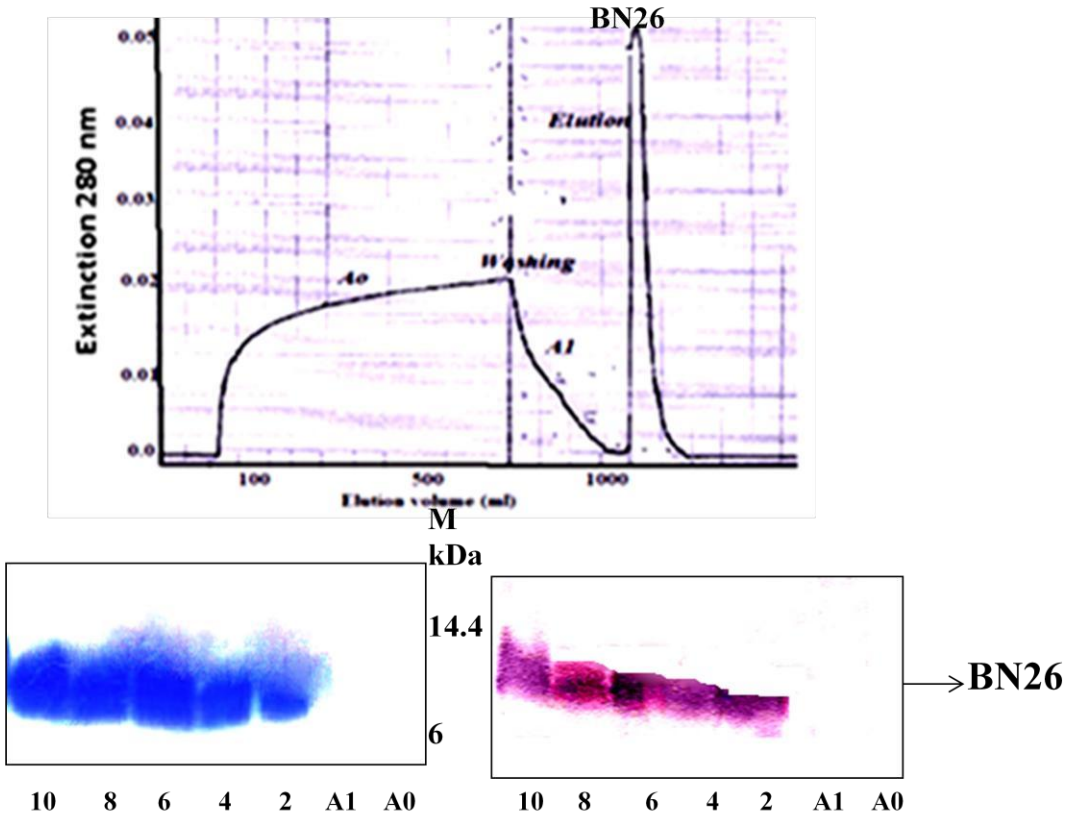


Figure 15: Purification of His-BN26 from yeast via reverse-phase chromatography using Amberlite XAD7. BN26 was detected as a single peak in the elution fraction as recorded by the chromatogram (A). Different fractions from BN26 protein application step (A0), washing step (A1) and different elution fractions (2, 4, 6, 8, and 10) from the protein peak. The collected fractions were neutralized with Tris base at pH 8. Then transfered in a UNIVAPO150H vacuum concentrator centrifuge overnight to evaporize the CH_3CN and dry the protein fractions. The dried protein fractions were dissolved with 50 µl sample buffer, of which 20 µl per gel lane was applied and separated electrophoretically. Subsequently, the proteins were transferred to a nitrocellulose membrane. The protein was separated by approximately 1 hour at 150 volts. The separated fractions were detected via Coomassie staining and Western blot (Anti-His).

Fig. 15 shows the purified BN26 analyzed by Coomassie stained polyacrylamide gel and Western blot analysis. The over expressed BN26 culture (1 L) was applied onto a small Amberlite reverse-phase chromatography column. In the chromatogram a single elution peak was detected. The fractions of the elution show a single band (8.7) between the 6 and 14 kDa markers on Coomassie stained SDS-polyacrylamide gels and western blots.

4.3 Isolation and purification of Cryoprotectin from cabbage

As described in Materials and Methods, hardened and frozen cabbage leaves were used for CPP isolation. The isolated CPP was precipitated by 60% ammonium sulphate solution. The precipitate can be stored for several months in the refrigerator. After centrifugation of 200 ml, the pellet was dissolved in 20 ml of 0.1% TFA adjusted to pH 2 and applied directly without desalting onto an Amberlite XAD7 column. The eluted proteins were neutralized with 1M Tris base to pH 8. Fig. 16 shows the purified CPP analyzed by Coomassie stained polyacrylamide gel and Western blot analysis.

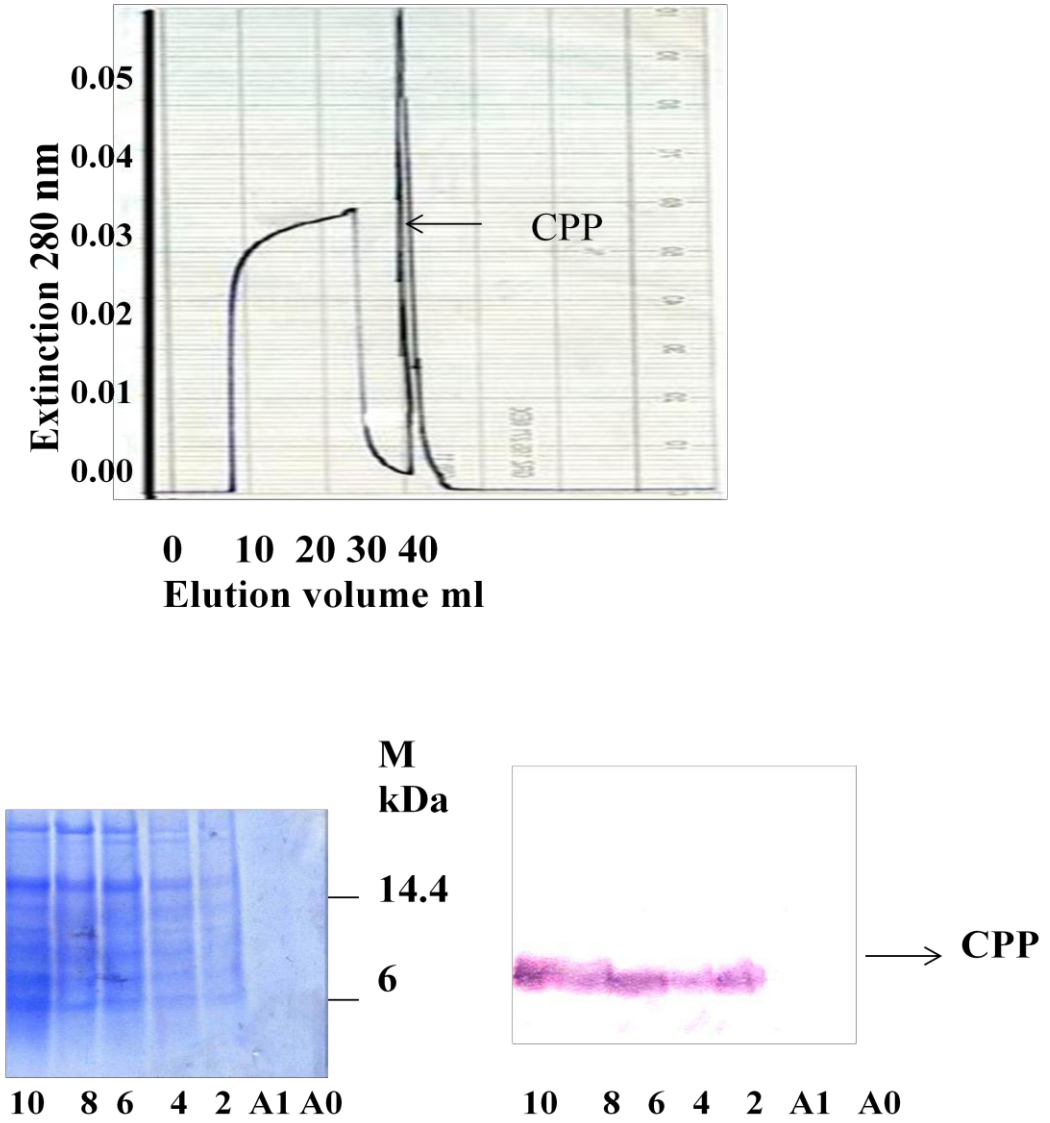


Figure 16: Purification of CPP crude extract via reverse-phase chromatography using Amberlite XAD7. Different fractions of CPP protein (A0, A1, 2, 4, 6, 8, 10) from application, washing and elution steps, respectively, were analysed. The collected fractions were neutralized with Tris base at pH 8, and in a UNIVAPO150H vacuum concentrator centrifuge remained overnight to evaporize the CH_3CN and dry the protein fractions. The dried protein fractions were dissolved with 50 µl sample buffer, of which 20 µl per gel lane was applied and separated electrophoretically. Subsequently, the proteins were transferred to a nitrocellulose membrane. The proteins were separated by approximately 1 hour at 150 volts. The separated fractions were detected via Coomassie staining and Western blot (Anti LTP).

4.4 Biochemical and biophysical analysis of BN26

The mode of action of cryoprotective protein can be established through a combination of biophysical and biochemical methods.

4.4.1 Secondary structure determination via circular dichroism (CD) spectroscopy measurements

Far-UV circular dichroism (CD) spectroscopy is used in one of the most successful applications of CD, the determination of the amount (or lack) of secondary structure composition of a protein because of its sensitivity to protein secondary structure (Greenfield, 1996; Sreerama and Woody, 2000a; Hennessey *et al.*, 1981, 2003). The ellipticity spectrum of IUPs has a large negative peak at around 200 nm and a value close to zero at 220 nm, distinct enough from that of ordered conformations to allow identification of partially or fully unstructured proteins (Nakayama *et al.*, 2008).

Proteins usually consist of more than one secondary structure element and the obtained spectra are more ambiguous because single absorptions of structural elements interfere with each other. Therefore, the spectra have to be computationally analyzed using a variety of computer algorithms, so that the approximate fraction of each structure type present in a protein can be determined (Sreerama *et al.*, 2001; Greenfield, 1996; Sreerama *et al.*, 2000; Sonnichsen *et al.*, 1992; Sreerama, 2004).

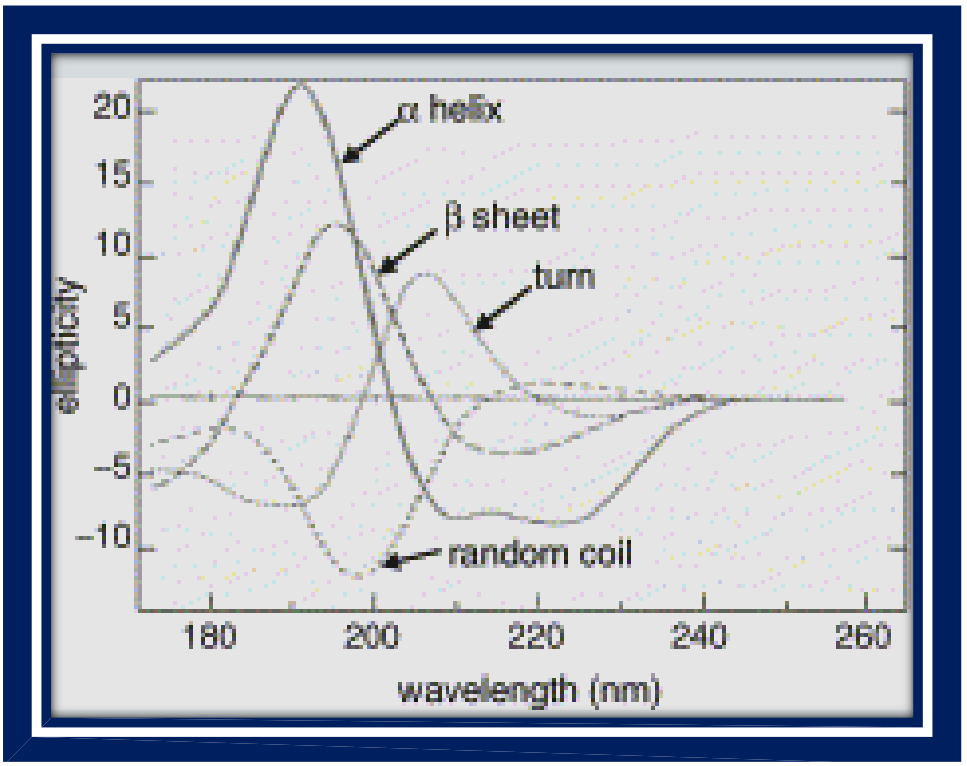


Figure 17: A typical far-UV CD spectrum of classical secondary structure elements from (Cabrita and Bottomley, 2004).

The ellipticity of BN26 was measured in degrees, when scanned from 260 nm to 186 nm. The measurement was performed in cooperation with Anja Thalhammer (PhD student in the Max Planck Institute for Molecular Plant Physiology Golm, AG Hincha). Fig. 18 shows that BN26 exhibited far-UV CD spectra in water typical for an unfolded polypeptide with a large negative peak at ~ 200 nm. However after drying the protein is prone to adopt a helical structure, as indicated by the minor negative peak at ~220 nm. The small ellipticity at ~220 nm unlike the zero or positive values shown by unfolded polypeptides indicated the presence of some β-sheet structure.

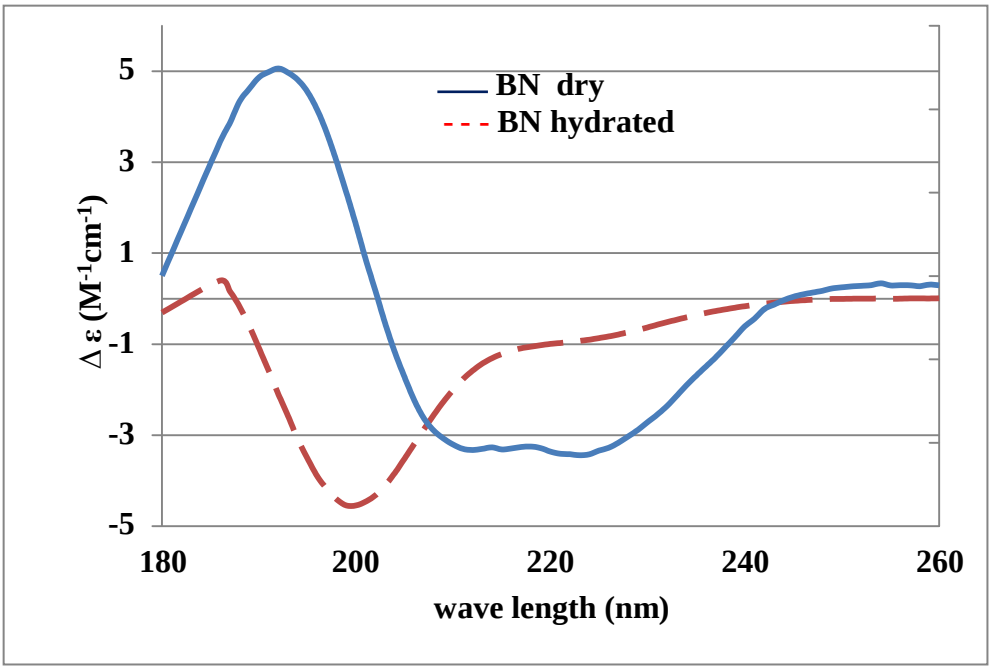


Figure 18: CD spectra of BN26 in dry and hydrated state. The spectra were measured in 0.1 mm Suprasil quartz cuvettes (liquid) or as dry protein films on CaF_2 windows from 260 to 180 nm. The analysis showed the CD spectra of BN26 in the dry (blue) and hydrated (red) state. BN26 shows a highly unordered structure when hydrated and gained mainly α-helical structure when dried. Data represent the means from measurements on three different samples.

Secondary structure estimation was done using CDPro software for computantional analysis of the data as described in Materials and Methods. The analysis revealed that BN26 was 65% unstructured in water. However, upon drying the α-helix fraction strongly increased from 10% to 50% and the percentage of unordered structure decreased from 60% to 20%, as shown in Fig. 19. In the dry state the β-sheet fraction was estimated to be ~20%.

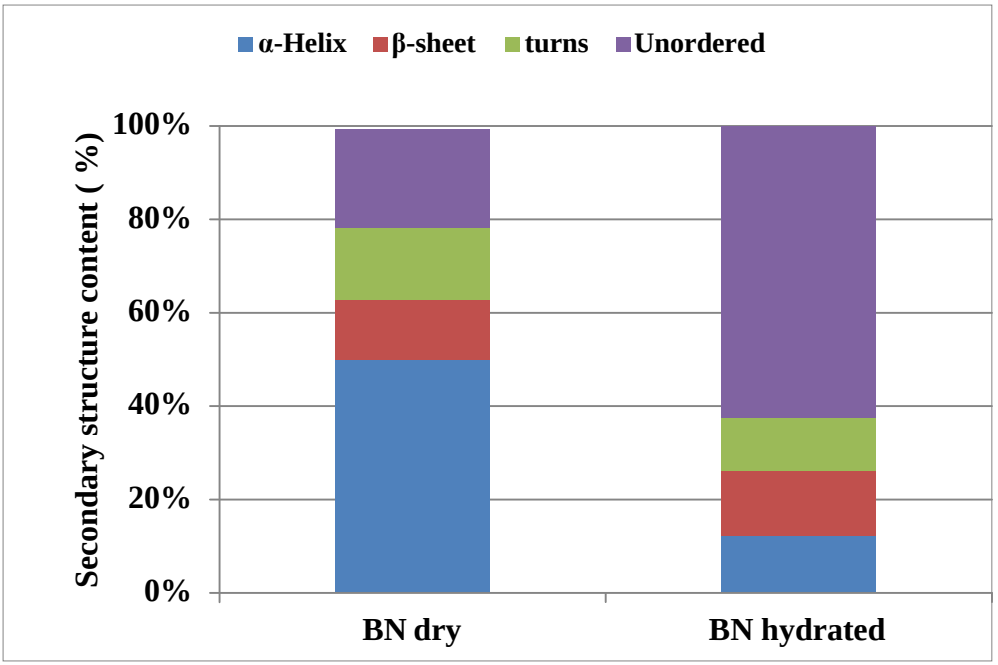


Figure 19: Relative content of different secondary structure elements in BN26 in the dry and hydrated state. The result showed that BN26 is mainly unordered in the hydrated state but prone to adopt α-helical structure when measured in the dry state. The calculation with CDPro software showed that BN26 gains a high ratio of α-helical structure in the dry state. However, upon hydration, the α-helical fraction was strongly reduced and the unordered fraction increased. Data represent the means from measurements on three different samples.

Structure-inducing agents such as trifluoroethanol (TFE) are known to promote the helix structure (Sonnichsen *et al.*, 1992). The promotion of the ordered state in BN26 was examined upon the addition of 50% (TFE) which increased the helix structure up to 55% (Fig. 20). TFE is not a physiological compound, but it may mimic the structural changes induced by binding to naturally occurring ligands.

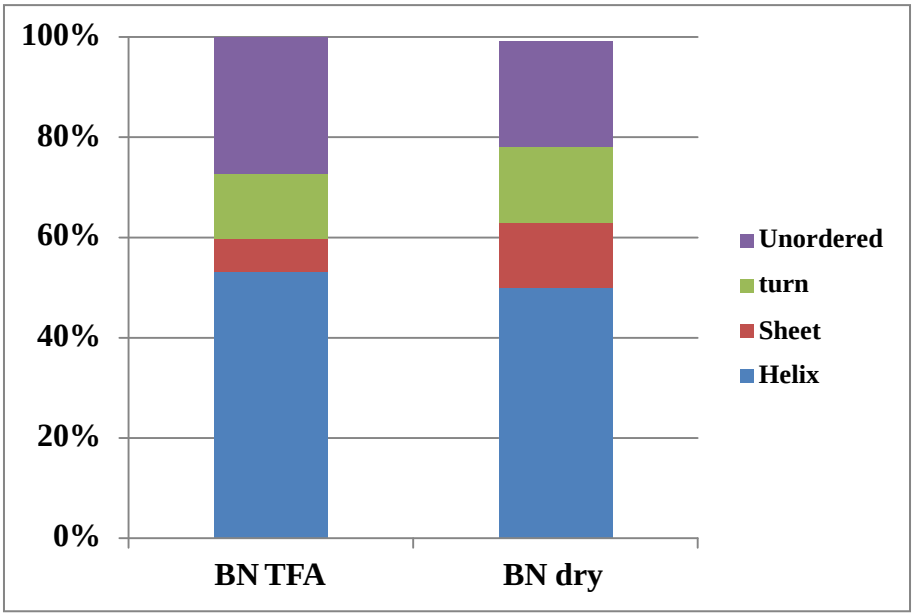


Figure 20: Induction of the helical content by TFE. TFE induced the helical content of BN26 to 55% as in case of dehydration. The calculation with CDPro software showed that BN26 gains a high ratio of α-helical structure under the induction of TFA. However, upon addition of TFA, unordered fraction was strongly reduced and the α-helical fraction increased. Data represent the means from measurements on three different samples.

4.4.2 Protease sensitivity assay

Due to their open and flexible conformational state which results from the lack of secondary structure, IDPs are exceptionally sensitive to proteolysis than globular proteins (Uversky, 2002; Tompa, 2005).

Unstructured proteins or (IDPs) are in whole or in part, in contrast to globular proteins, ensembles of flexible molecules, unorganized and without a defined three-dimensional structure. According to these properties, the proteins are referred to as natively unfolded, intrinsically unfolded (IUP), intrinsically unstructured or intrinsically disordered (Uversky, 2002; Tompa, 2005).

In order to investigate the sensitivity to proteolytic degradation, an *in vitro* assay to test the flexibility of BN26 was performed. In this test 100 µg BN26 and lysozyme were incubated with the serine protease trypsin that predominantly cleaves proteins after basic lysine and arginine residues. Digested BN26 and lysozyme were visualized by SDS-PAGE. Fig. 21a shows that BN26 is sensitive to protease degradation whereas the globular protein lysozyme remains intact under the same conditions.

BN26 is claimed to have greater flexibility to protease degradation than lysozyme. This is suggested to be due to the lack of structural elements, BN26 is more successible to proteolysis. This effect is not caused by a massive change in Lys/Arg content. Both proteins contain 10-20% of positively charged amino acids.

The amino acid backbone of globular proteins is resistant to proteolytic attack because it is to a big extent hidden from the surface for their well-defined three-dimensional structure. Due to the lack of a compact, globular fold, unstructured proteins resemble the denatured states of globular proteins (Tompa, 2003). For this reason, proteolytic degradation of lysozyme as a folded protein require denaturation prior to proteolytic degradation (Suskiewicz *et al.*, 2011). Freezing of BN26 and lysozyme pior to incubation with trypsin revealed different behaviour towards proteolytic activity (Fig. 21b). In case of BN26 it remains intact as it exhibits its structural form under freezing stress. On the other hand Lysozyme was digested as it was denatured (Fig. 21b).

.

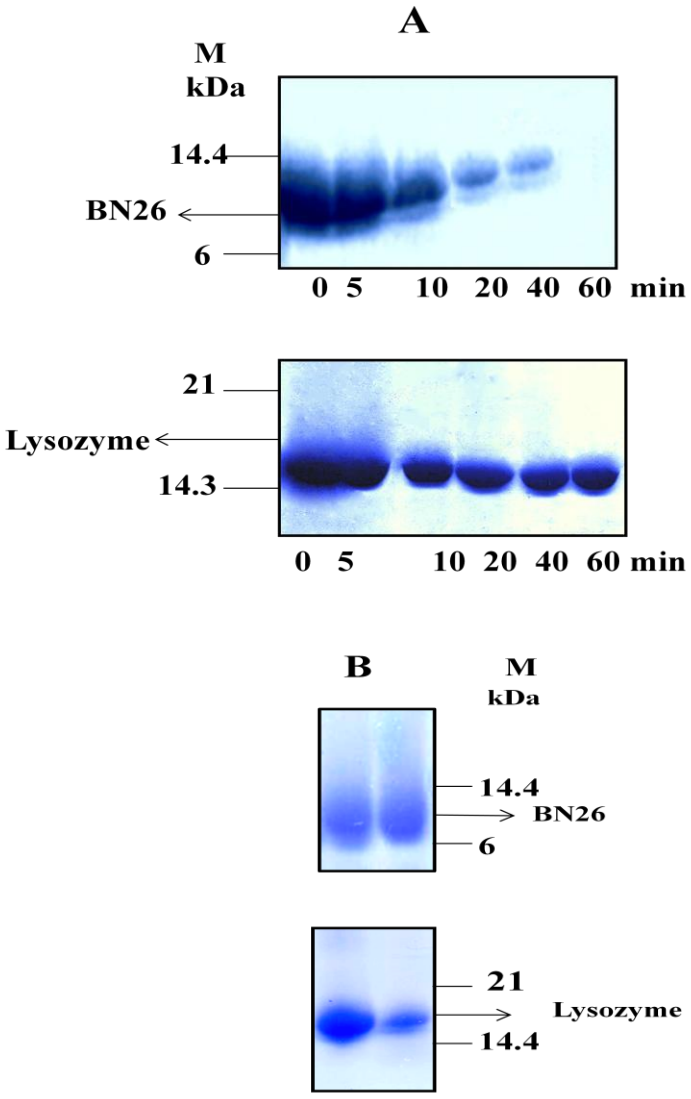


Figure 21: Protease sensitivity assay. (A) 100 µg of BN26 and Lysozyme were incubated with 100 ng trypsin for 1h at 37 ºC over the time intervals. Lysozyme was used as globular protein control, showing resistance to proteolysis. Aliquots were removed over a time course and the reaction was stopped by the addition of SDS-PAGE loading buffer and immediate boiling for 5 min and analyzed by SDS-PAGE by running on a 10-20% polyacrylamide gradient gel. After 60 minutes no uncleaved BN26 was detectable in contrast to lysozyme, which was still intact under the same conditions. (B) The lower panel shows the effect of digestion after freezing to denature lysozyme and freeze-stressed BN26 and incubation with 100 ng of trypsin for 1h at 37°C. Then 2 aliquots were removed and the reaction was stopped by the addition of SDS-PAGE loading buffer and immediate boiling for 5 min and analyzed by SDS-PAGE by running on a 10-20% polyacrylamide gradient gel.

4.4.3 Cryoprotective activity of BN26

It is well-known that the plasma membrane and thylakoids are damaged during freeze–thaw cycle, both *in vivo* and *in vitro* (Steponkus, 1984; Hincha and Schmitt, 1992a). For this reason, the plasma membrane and the chloroplast thylakoid membranes are the best characterized model systems to study the molecular basis of freezing tolerance in *vitro* (Hincha, 2008). Thylakoid membrane vesicles isolated from non-hardened spinach have been used in this study as a model membrane system (Hincha *et al.*, 1996). Freezing damage leads to leakage of thylakoid content leading to collapse of the vesicles. Therefore, evaluation of the freeze-thaw damage of thylakoid membranes can be measured in a volumetric assay as a decrease in packed thylakoid volume (Hincha and Schmitt, 1992). This test was used to investigate the cryoprotective activity of BN26.

As positive controls cryoprotectin (CPP) isolated from cold hardened *Brassica oleracea* and COR15A were used. For CPP a protection of thylakoids from freeze-thaw damage was previously shown (Hincha *et al.*, 1989, 1996a). As negative controls BSA, lysozyme and the *Pichia* X33 [wild type] protein were used.

Fig. 22 shows the cryoprotective activity of BN26 and other tested additives. Cryoprotective protein activity can be quantified as the conservation of thylakoid volume which means the maintenance of thylakoid integrity after a freeze-thaw cycle. The thylakoid volume of non-treated thylakoids was set to 100% cryoprotection.

The results showed that cryoprotectin (72%) exhibits high cryoprotectitive activity compared to all tested additives. BN26 also affords (51%) cryoprotective activity which is better than COR15A (28%). BSA and lysozyme showed a very small extent of cryoprotective activity with (16%) and lysozyme (5%), respectively. However, this small cryoprotective activity was over the level of *Pichia* X33 which showed virtually no cryoprotective activity.

BN26	**51.00**
COR15A	**28.00**
BSA	**16.00**
CPP	**72.00**
Lysozyme	**5.000**
Pichia x33	**0.4000**

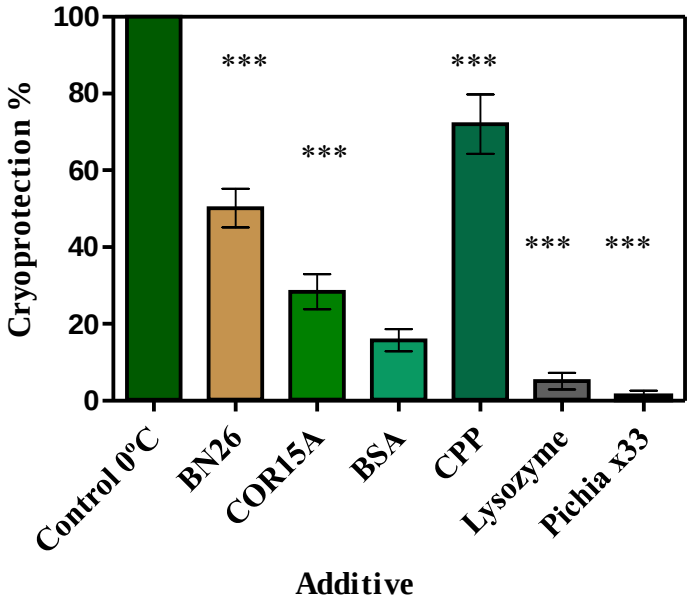


Figure 22 : Cryoprotective activity of BN26. Cryoprotective activity was determined as thylakoid volume by hematocrit centrifugation after freeze-thaw cycle of BN26 and the tested proteins. BN26 and different proteins were suspended in 10 mM Sucrose, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and desalted over a NAP5 column. The protein samples were taken from a 1 mg/ml protein stock solution and mixed with 100 µl Thylakoid suspension (from 1 mg of chlorophyll/ml). The data are presented as means ±SD of six experiments with total of 5 samples and 3 replicas. Asterisks indicate the level of significance in comparison to BSA as revealed by Student's *t*-test (∗$P < 0.05$, ∗∗$P < 0.01$, ∗∗∗$P < 0.001$). The calculated mean value for each additive is marked in the table over the bar diagram.

4.4.4 Cryoprotective activity of BN26 is correlated to protein concentration

To characterize the cryoprotective activity of BN26 in more detail different concentrations of BN26 were tested in the above described thylakoid freezing test. The result showed that cryprotective activity of BN26, CPP and BSA is dependent on protein concentration. Fig. 23 shows that the cryoprotective activity of BN26 increased gradually with concentration till it reached saturation at protein concentration of 500 µg/ml. However, the cryprotective activity result of CPP shows that it is dependent on protein concentration. Bovine serum albumin (BSA) only provided a very little degree of cryoprotection around 15% even at high concentrations.

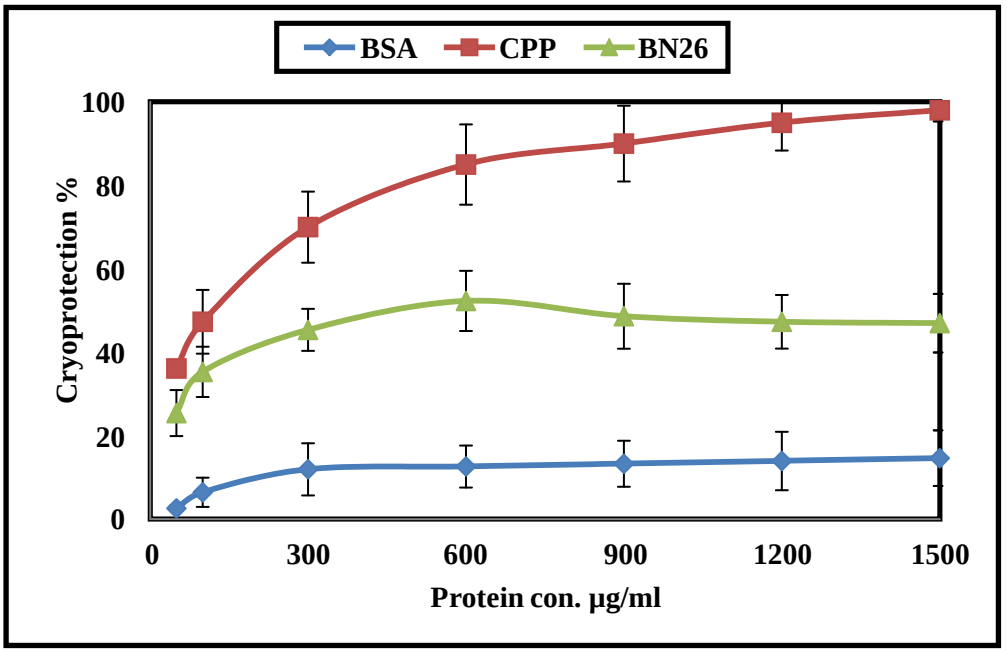


Figure 23: Cryoprotective activity of BN26 is protein concentration-dependent.

BN26, CPP and BSA were suspended in 10 mM sucrose, 1 mM $MnCl_2$, 1 mM $CaCl_2$ and desalted over NAP5 column. 100 µl of different concentrations of BN26, CPP and BSA from 0-1.5 mg/ml were mixed with 100 µl thylakoidsuspension from 1 mg of chlorophyll/ml. Cryoprotective actvity was measured by determination of thylakoid volume after freezing by hematocrit centrifugation. The data are presented as means ± SD of six experiments with a total of 3 samples and 3 replicas.

4.4.5 Cryoprotective activity in the presence of combinations of different additives

The idea was to find out if the interaction between BN26 and other cryoprotective proteins have influence on the cryoprotective activity. For this aim, BN26 was mixed with either COR15A or CPP and with both proteins. Cryoprotective activity of the protein samples was tested for each individual protein and compared for the cryoprotective activity of different protein mixtures.

Fig. 24 shows that the cryoprotective activity of BN26+CPP is enhanced when the two proteins are in combination. According to the results the effect was more pronounced between BN26 and BN26+CPP (50 % to 80%) in comparison to the cryoprotective activity of each single protein. In the same manner a higher cryoprotective activity could be observed if we compare COR15A and COR15A+BN26 (30% to 50%). Interestingly the cryoprotective activity is comparable between the samples containing just BN26 or BN26 mixed with COR15A. In contrast, the activity of CPP decreased when combined with COR15A. On the other hand the combinations of the three proteins inhibited the cryoprotective activity of BN26, COR15A and CPP compared to their individual activities. Tab. 9 shows the significance differences between the activity of different protein combinations compared to the activity of each single protein.

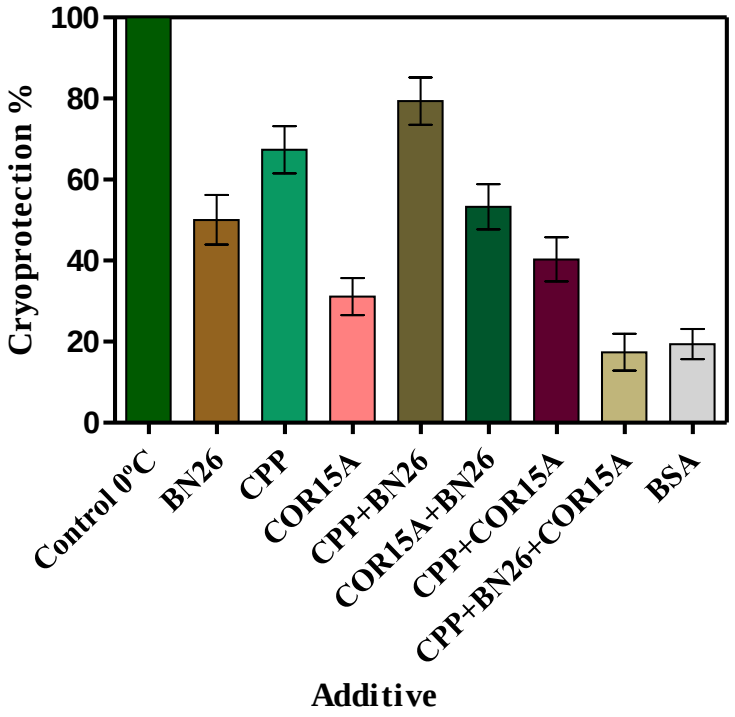


Figure 24: Cryoprotective activity of combinations of BN26 and diffrent additives.

Cryoprotection activity of BN26 and different additives in comparison to individual activity of each protein alone. 100 µl of each the protein samples from a 1 mg/ml protein stock solution alone or 50 µl for protein mixtures were mixed with 100 µl Thylakoid suspension (from 1 mg of chlorophyll/ml). The data are presented as means ± SD of six experiments with a total of 3 samples and 3 replicas. Asterisks indicate the level of significance compared to each protein alone as revealed by Student's *t*-test (∗$P < 0.05$, ∗∗$P < 0.01$, ∗∗∗$P < 0.001$).

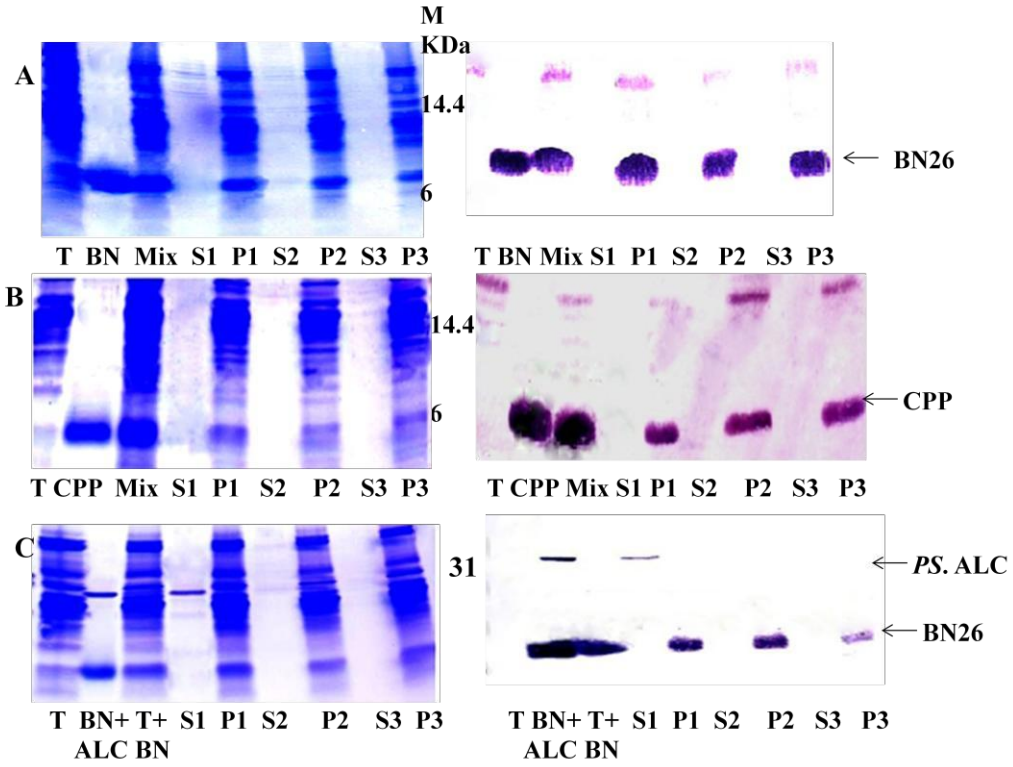
Table 14: Statistic analysis using the Student's *t*-test shown are significance differences with three P-values thresholds $P < 0.05$, $P < 0.01$, $P < 0.001$.

	BN26	**CPP**	**COR15A**	**BN26+CPP+COR15A**
BN26+CPP	$P <$ 0.0001	$P <$ 0.0001	not tested	$P <$ 0.0001
BN26+COR15A	not significant	not tested	$P <$ 0.0001	$P <$ 0.0001
BN26+CPP+COR15A	$P <$ 0.0001	$P <$ 0.0001	$P <$ 0.0001	not tested
CPP+COR15A	not tested	$P <$ 0.0001	$P <$ 0.0001	$P <$ 0.0001

4.4.6 BN26 binding to the thylakoid membrane

Binding of BN26 to the thylakoid membrane was studied in this investigation as a possible mechanism of cryoprotection. It has been previously reported that the effect of cryoprotectin (CPP) in the cryoprotection mechanism is based in part on the stable binding of CPP to the thylakoid membrane (Sror *et al.*, 2003). The binding of cryoprotectin to thylakoid membranes reduces the fluidity of the membrane. To investigate the possible binding of BN26 to thylakoids, thylakoids from non-hardened spinach leaves were incubated with His-BN26 or CPP (positive control) or *Pseudomonas syringae* allantoicase (ALC) His-tagged protein expressed in *E.coli* (Werner *et al.*, 2010) as a negative His-tagged protein. Then each of the previous protein mixtures was subjected to three rounds of centrifugation and resupension of the sedimented pellet in protein–free solution (as described in Materials and Methods). Samples were taken from each round of sedimentation for both supernatant and pellet and analyzed by SDS-PAGE and Western-Blot analysis. Fig. 25A shows the binding ability of BN26 to the thylakoid membrane. SDS-PAGE and western–blot analysis of the supernatant and the pellet from each washing step shows that the supernatant was always free of BN26. The BN26 protein (8.7 KDa) was almost exclusively detected in the pellet and was permanently resistant to the repeated washing steps. That means that the BN26 protein binds to the thylakoid membranes.

CPP binding ability (Fig. 25B) was also detected with Western-Blot-analysis in the pellet in agreement with the result of Sror *et al.*, (2003). Fig. 25C shows an example for another His-tagged Protein *P.s.* (ALC) as a negative control which appears in the first supernatant and, in contrast to BN26, is never detected in the pellet.



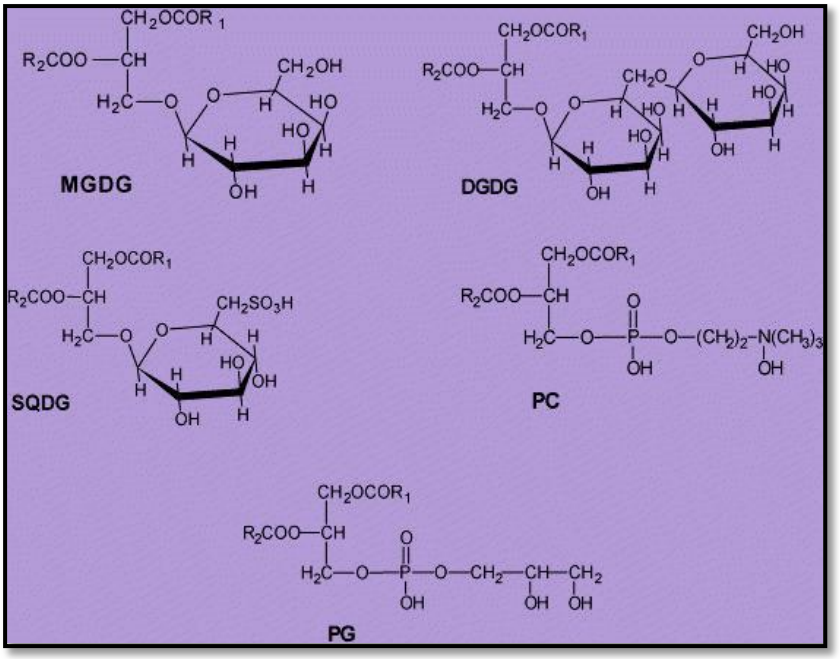
]

Figure 25: BN26 binds to thylakoid membranes. 500 µl of BN26 protein solution (100 µg) was mixed with 500 µl thylakoids in eppendorf (100 µg chlorophyll) and incubated on ice for 15 min. The mixture was subjected to three rounds of centrifugation (14,000 rpm for 5 min) and resupension in protein free solution. Aliquots from the supernatant and the pellet were analyzed by SDS-PAGE and Western-blot. BN26 protein remained bound to the thylakoid membrane (A) and was resistant to several washing steps similar to CPP which was used as a positive control (B). When a mix of *P.s.* (ALC) and BN26 was tested, only BN26 remain bound to the thylakoid membrane (C). Thy: thylakoids; S: Supernatant; P: Pellet; BN: BN26; *P.s.* (ALC): (-ve control protein); CPP: Cryoproectin (positive control protein); Mix = mix of protein and thylakoids.

4.4.7 Interaction of the BN26 protein with liposomes in a thylakoid membrane model

Native thylakoid membranes are composed of approximately 50% proteins and 50% lipids by weight. The dominating species is the uncharged non-bilayer glycolipid monogalactosyldiacylglycerol (MGDG; Fig. 26a) with 57% by weight (Webb *et al.*, 1991; Lee, 2000). The second most important lipid is digalactosyldiacylglycerol (DGDG), which represents 27% of the total lipid (Dome, 1990). The remaining classes consist of the anionic bilayer lipids sulphoquinovosyldiacylglycerol (SQDG) (7%) and phosphatidylglycerol (PG) (7%) which are negatively charged bilayer lipids. Both SQDG and PG represent the acidic lipids. The unusual chemical structures of MGDG and DGDG give them unusual packing properties. The ways in which lipids pack together depends on their shape. A simple phospholipid such as a phosphatidylcholine has a cylindrical shape; the cross-sectional area of the phosphocholine headgroup is about the same as that of two fatty acyl chains (Fig. 26b). Cylinders are ideally suited for packing side by side in a bilayer. However, not all lipids have a cylindrical shape.

A



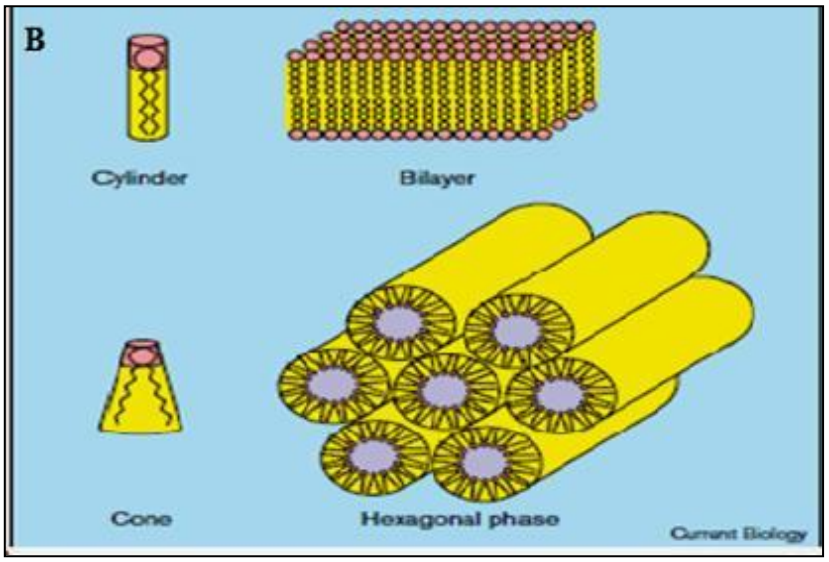


Figure 26: Schematic representsation of the structures of lipids in thylakoid membrane from (Yao *et al.*, 2006) (**A**) and the concept of lipid shape and its effect on lipid packing (**B**) from (Lee, 2000).

4.4.7.1 Maintenance of liposome membrane integrity after freezing

4.4.7.1.1 Carboxyfluorescein leakage from liposomes after freezing

Liposomes were used as simplified model for biological membranes. Liposomes mimicking thylakoid membrane (Webb, 1991) were used. This is common to modify the composition of the model membrane to analyze interactions of membranes with various macromolecules (Bangham, 1972). Previously, the possible roles of low-temperature induced polypeptides (COR6.6 and COR15A) in membrane stabilization were determined (Uemura *et al.*, 1996; Webb *et al.*, 1996).

Leakage experiment was performed to gain an insight on liposome-stabilizing properties of BN26. Leakage of solutes from the liposome interior can occur with the removal of water during freezing.

When the fluorescent dye carboxyfluorescein (CF) a fluorescent staining dye is encapsulated into liposomes, self-quenching occurs due to the highly concentrated dye inside liposomes and no fluorescence is emitted. However, upon leakage this effect vanishes by the decrease of CF-concentration while distributed in the surrounding medium. Strong fluorescence is a sign of liposome damage. Leakage can be measured as the ratio of fluorescence measured before and after liposome permeabilization with 1% of the detergent TritonX-100, which disrupts the membranes and leads to complete leakage.

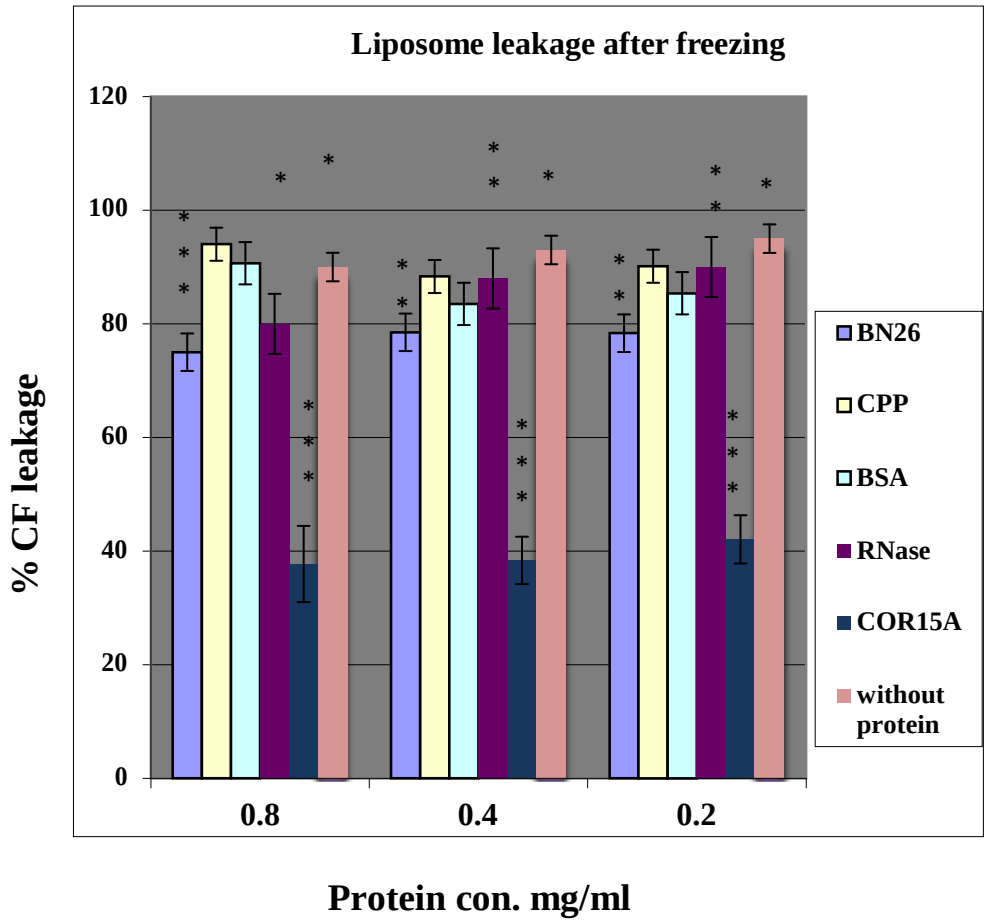


Figure 27: CF Leakage from liposomes (40% MGDG, 30% DGDG, 15% EPG, and 15% SQDG) after freezing and thawing. The effect of BN26 on the freeze-induced leakage of liposomes was measured by freezing liposomes at -20ºC for 3h with BN26, different control proteins and in buffer without protein. Leakage values represent means ± S.D from three parallel samples. Asterisks indicate the level of significance in comparison to BSA as revealed by Student's *t*-test (∗$P < 0.05$, ∗∗$P < 0.01$, ∗∗∗$P < 0.001$).

Fig. 27 shows that BSA, RNase and cryoprotectin (CPP) neither reduced nor prevented freeze-induced leakage of liposomes. This result suggests that these proteins do not have cryoprotective effects on liposomes. RNase was used as control because of its comparable size with BN26. BN26 showed a moderate cryoprotective effect (28%) on liposome leakage. In contrast to BN26, highly potent protection against CF leakage by COR15A could be shown on liposomes. This was indicated by improved retention of the fluorescent dye after thawing. This behavior of BN26 and COR15A in liposome protection distinguishes between them regarding the 69% identity in the coding regions of their genes. As BN26 did not show the same membrane-stabilizing effect as COR15A which was able to increase stability to liposomes during freezing in a high extent.

4.4.8 Refolding activity of BN26

In order to define the basic mechanism by which BN26 could facilitate refolding of denatured proteins, the effect of BN26 on luciferase refolding was tested. Luciferase was inactivated by incubation at 42 °C in the presence and absence of BN26, COR15A, BSA and the buffer without any additive. Reactivation was monitored after heat treatment (42 °C) for a time period of 60 min at 30 °C with and without ATP. Then luminescence was measured over time to test the ability of the proteins used in recovering the luciferase activity.

As shown in Fig. 28 BN26 effectively increased the recovery of luciferase activity from the denatured state. BN26 was able to help luciferase to regain up to 70% of its activity over 1 hour either in the presence or absence of ATP. In contrast, neither COR15A nor BSA was able to refold the enzyme in agreement with what has been previously reported by Nakayama *et al.,* (2008). This implies that COR15A has no chaperone activity either in the presence or absence of ATP. BN26 thus showed a positive effect on luciferase reactivation whereas COR15A showed no effect under the same conditions. Chaperone is a term used to explain the way by which the protein bind to nonnative forms of other proteins to maintain or regain their native conformation (Gatenby and Viitanen, 1994; Welch and Brown, 1996).

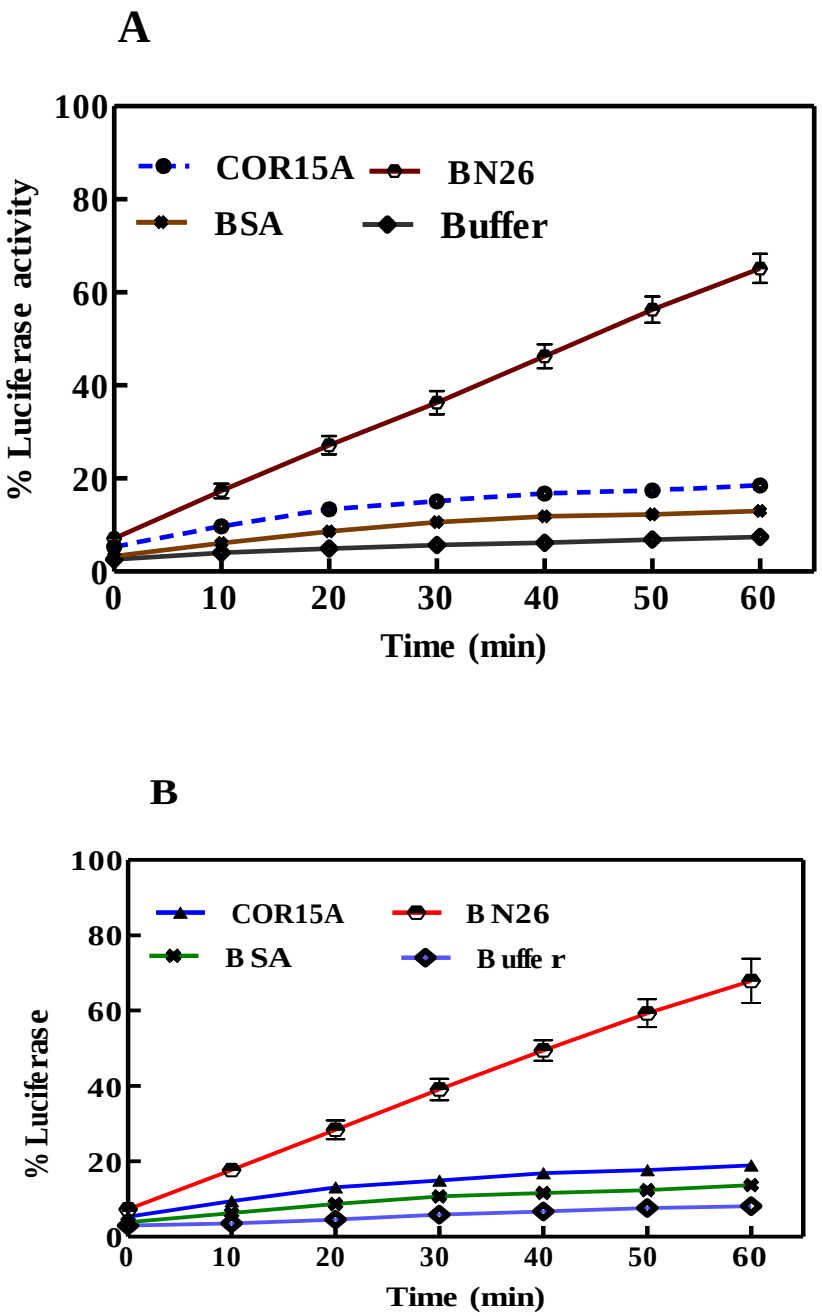


Figure 28: Refolding activity of BN26. Luciferase was denatured in the presence of 1 µM (0.087 mg/ml) BN26 (circles) or 1µM (0.097 mg/ml) COR15A or 1 µM (0.066 mg/ml) BSA (crosses) or without additives (Buffer; diamond) at 42 ºC for 10 min, then cooled to 30 ºC to initiate refolding in the presence of 2.5 mM ATP (**A**) and in the absence of ATP (**B**). Luciferase activity prior to heat denaturation was defined as 100%. Data points represent averages of (n=9) ± SD for three independent experiments in triplicate.

4.4.9 *In vitro* assays to measure lactate dehydrogenase stabilization under different stresses

4.4.9.1 Cryoprotection of LDH against freeze-thaw inactivation

The freeze-lability of lactate dehydrogenase (LDH) has led to the establishment of an *in vitro* assay using it as a reporter enzyme to assess the effectiveness of a "cryoprotectant" in preventing freeze denaturation (Santarius, 1982). In this assay the ability of compounds to prevent denaturation of the freeze-labile enzyme LDH was determined following a freeze-thaw stress. This assay was previously used to determine the cryoprotective properties of COR15A in *Arabidopsis thaliana* (Lin and Thormashow, 1992a). In both instances, the peptides were capable of preventing the freeze-denaturation of LDH.

In the present assay, BN26 affords virtually 97% protection of LDH from freeze-denaturation (Fig. 29). Equivalent concentrations of BSA, COR15A, and cryoprotectin, lysozyme, sucrose, and lactate dehydrogenase without cryoprotectant were used as controls. BN26 could retain up to 95% of the lactate dehydrogenase activity of untreated samples after one freeze-thaw cycle (Fig. 29). Cryoprotectin and COR15A could maintain 70% and 85% of the enzyme activity, respectively (Fig. 29). BSA only preserved 50% of the enzyme activity. Sucrose could not protect lactate dehydrogenase from freeze-induced inactivation. The globular protein Lysozyme, which is unrelated to stress responses, did not show any protection under the same conditions. Without the addition of a cryoprotectant, a freeze-thaw cycle resulted in a dramatic decrease ~ 90% of LDH activity. The empty vector (*Pichia* X33 [wild type]) showed virtually no protection. Therefore, in this assay, BN26 does appear to have effective cryoprotective properties and was able to stabilize lactate dehydrogenase against freeze- induced inactivation.

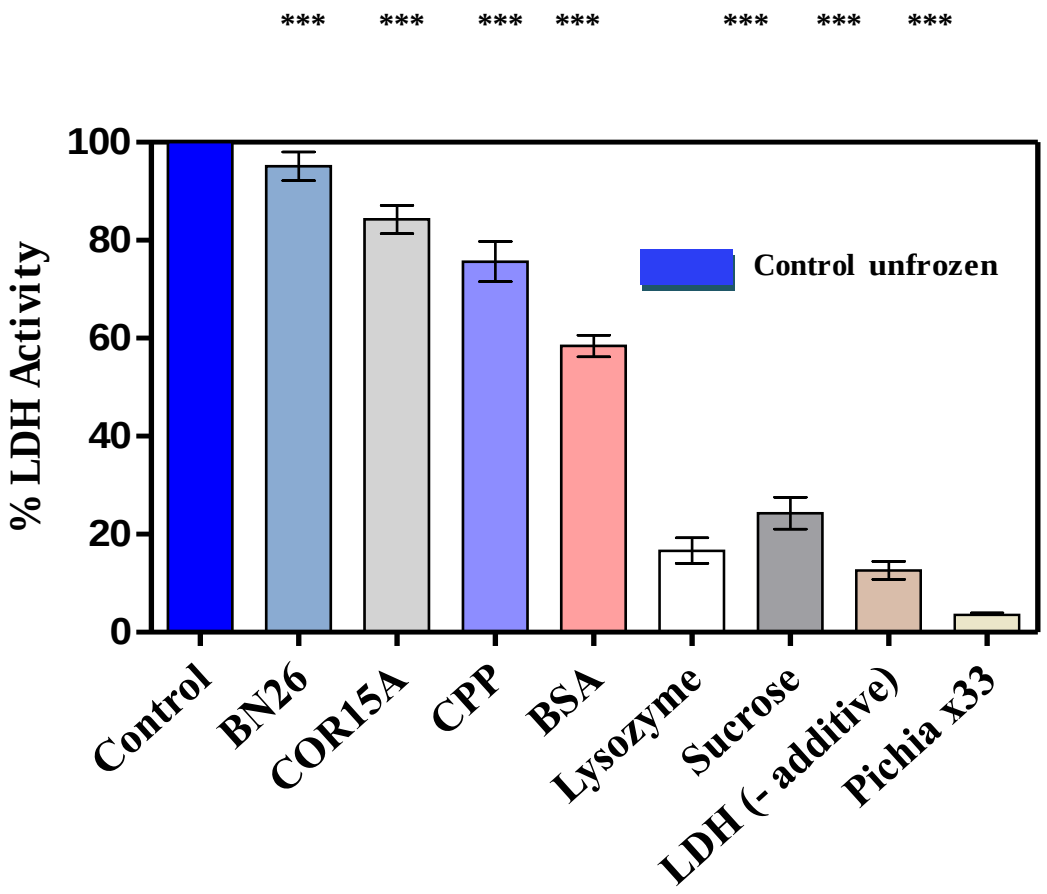


Figure 29: Cryoprotective of LDH by BN26. An LDH 0.5 µM solution was frozen at -20 °C for 24 h with 5 µM BN26, other tested additives except for sucrose (50 µg/ml) and buffer alone without additive in 10 mM Potassium phosphate buffer pH 7.5. The activity of unfrozen LDH is set as 100% activity. Residual LDH activity was calculated as the percentage of the activity of the unfrozen control before treatment. The data represent the ratio of the pretreatment enzyme activity. Values are the means of (n=9) ± SD of 3 independent experiments in triplicate. The asterisks represented the statistical difference between samples compared to lysozyme using Student's *t*-test (*$P \leq (0.05)$, **$P \leq (0.01$,*** $P \leq (0.001)$.

4.4.9.2 Cryoprotection of LDH against inactivation by repeated freeze-thaw cycles

To better visualize the cryprotective ability of BN26 on LDH activity, the assay was modified, performing several freeze-thaw cycles. The assay was done in the presence of BN26 and a set of control agents (COR15A, BSA, cryoprotectin, sucrose, lysozyme and the [Pichia X33]) wild type proteins (Fig. 30).

Fig. 30 shows that unprotected lactate dehydrogenase (without additives) had no measurable activity after one to five freeze-thaw cycles. However, BN26 showed a considerably high degree of cryoprotection of LDH activity (Fig. 30). After three freeze-thaw cycles the samples containing BN26 retained more than 65% of the initial LDH activity. COR15A also afforded a high protection capacity of LDH activity (Fig. 30). COR15A maintained 50% of the initial LDH activity after three freeze-thaw cycles.

Cryoprotectin maintained 35% of the LDH activity after three freeze-thaw cycles. BSA, with 20% residual LDH activity after freeze-thaw, showed a lower maintenance of LDH activity (20%) compared to the previous proteins. Lysozyme did not protect LDH from freeze-induced loss of activity. Sucrose showed a very slight protection but more than 18% of the activity was lost in buffer (without any additives) after one to five freeze-thaw cycles. The empty vector (i.e *Pichia* wild type without BN26 (*Pichia pastoris* x33) showed virtually no protection of LDH in comparison with the buffer alone. BN26, COR15A, BSA and cryoprotectin did not show a high protective effect on LDH activity after five freeze-thaw cycles. However, BN26 was the best as it still retained 30% of LDH activity. All the other tested additives showed virtually no protection after five freeze-thaw cycles.

*** ** *** *** *** ** **

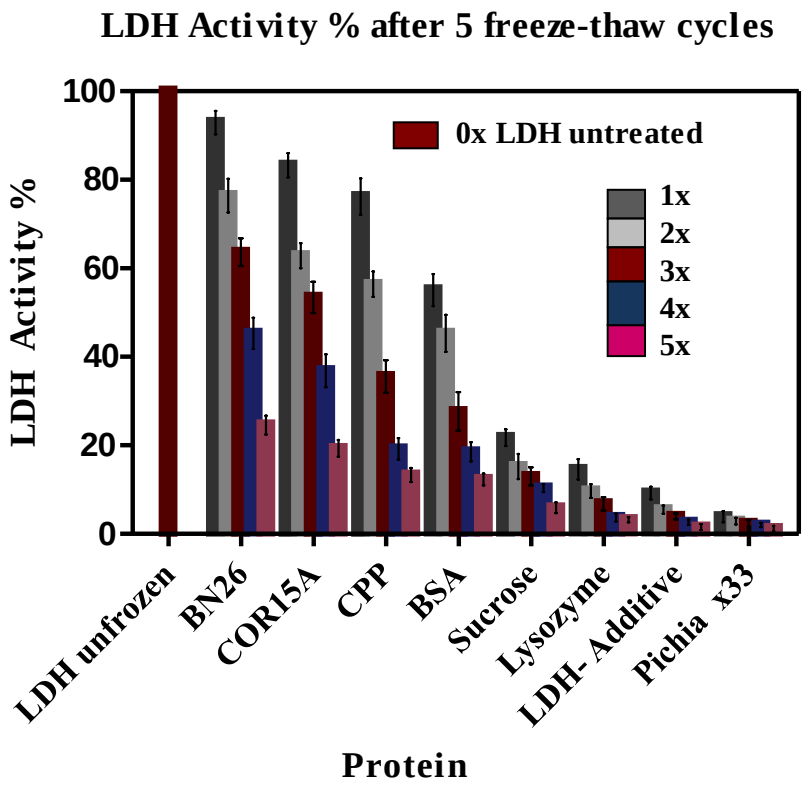


Figure 30 Cryoprotection of LDH by BN26 aftetr 5 freeze-thaw cycles. Solutions LDH alone 0.5 µM (no additive) or in the presence of 5 µM of BN26 or control additives (COR15A, Cryoprotectin, Lysozyme and empty vector control [Pichia x33]) but for Sucrose, BSA (50 µg/ml), in 10 mM potassium phosphate, pH 7.5 were exposed to five cycles of freezing (immersion in liquid nitrogen for 30 s) and thawing (immersion in water bath at 4 °C for 5 min). Residual activity of LDH after each freeze-thaw cycle was measured as percent of LDH activity before treatment (100%). Values represent the means of (n=9) ± SD of 3 independent experiments in triplicate. The asterisks represent the statistical difference between samples compared to lysozyme using the Student's *t*-test (*$P \leq (0.05)$, **$P \leq (0.01)$,*** $P \leq (0.001)$. CPP: cyoprotectin.

4.4.9.3 Dehydration stress protection assays

Lactate dehydrogenase is also known to be sensitive against dehydration. To gain insight into the dehydration stress protection by BN26, I have used an *in vitro* assay. In this assay lactate dehydrogenase, BN26 and the other tested proteins were dehydrated for 2 hours or overnight and then rehydrated to the original volume (Fig. 31).

Fig. 31 shows that LDH lost 90% of its activity after 2 hours of dehydration and the activity was completely abolished in the LDH solution without any additive and in sucrose or lysozyme solutions after 100 % dehydration overnight. The vector control protein [*Pichia* x33] showed virtually no LDH activity either after 2 h dehydration or after overnight dehydration.

It is interesting that BN26 exhibited significantly higher protective activity than all the other tested proteins (Fig. 31). LDH activity was retained at 90% of its initial activity after a water loss of 70% (2h dehydration) in the presence of BN26 protein. Of particular interest, BN26 protein exhibited significantly higher protective activity than all the other tested proteins when dehydrated for 24 h, as it still exhibited 70% of the initial activity.

In contrast, COR15A preserved only 30% of the LDH activity after 24 h dehydration but retained 70 % of LDH activity after 2 hdehydration. BSA can rescue approximately 20% of LDH's initial activity during the overnight dehydration.

Lysozyme, as a negative control, did not protect LDH activity from dehydration. These data indicate that BN26 has the ability to protect enzymatic activity from inactivation during dehydration, and this to a higher degree than COR15A.

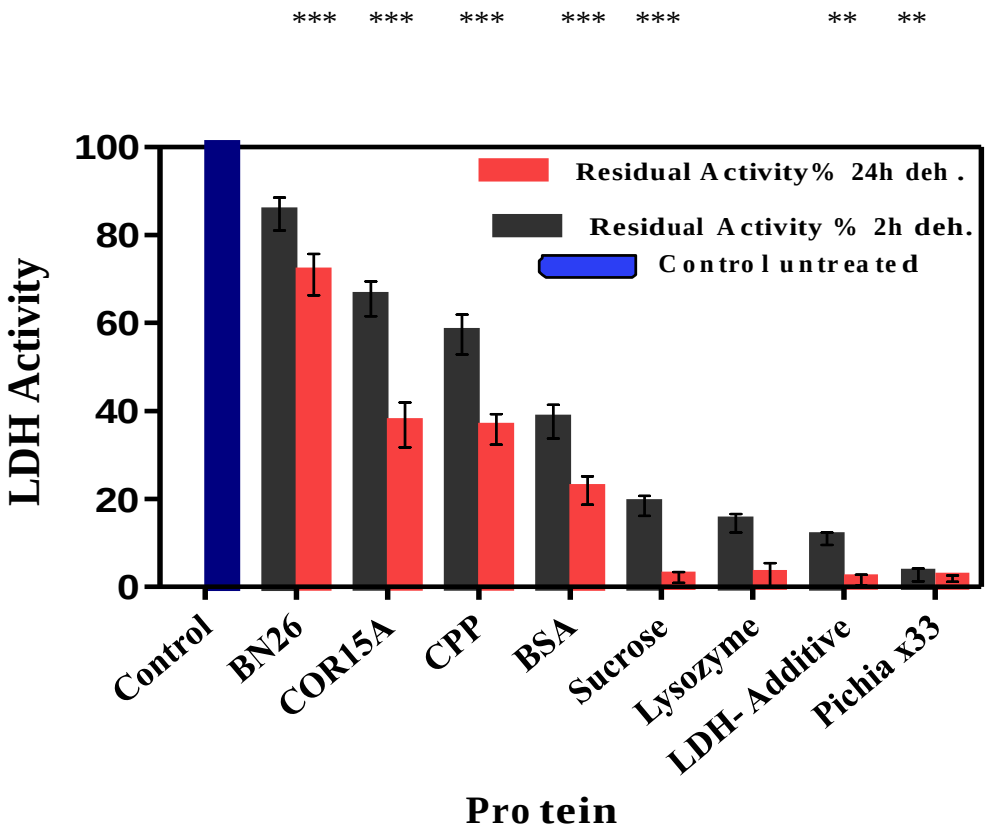


Figure 31: Protection of LDH by BN26 from dehydration. LDH alone and with BN26 or other tested proteins were either dehydrated for 2h (70%) or overnight (100%) using a Speed-Vac centrifuge at room temperature. The samples were then rehydrated to the original volume with water, and LDH activity was measured. LDH activity before treatment was defined as 100%. Residual LDH activity was measured as percent of LDH activity before treatment (100%). Values are the means of (n=9) ± SD of 3 independent experiments. The asterisks represented the statistical difference between samples compared to lysozyme using the Student's *t*-test (*$P \leq (0.05)$, **$P \leq (0.01$,*** $P \leq (0.001)$. CPP: cryoprotectin.

4.4.9.4 Effect of BN26 on LDH refolding activity

It is well known that IDPs (proteins with no secondary structure) have both RNA and protein chaperone activity (Tompa and Csermely, 2004). To investigate if BN26 can recover lactate dehydrogenase activity or not, BN26, CPP and BSA were added to the denatured enzyme. Fig. 33 shows the ability of BN26 to recover LDH activity. BN26 was able to recover the activity of BN26 up to 85%. However, neither CPP nor COR15A were able to recover LDH activity.

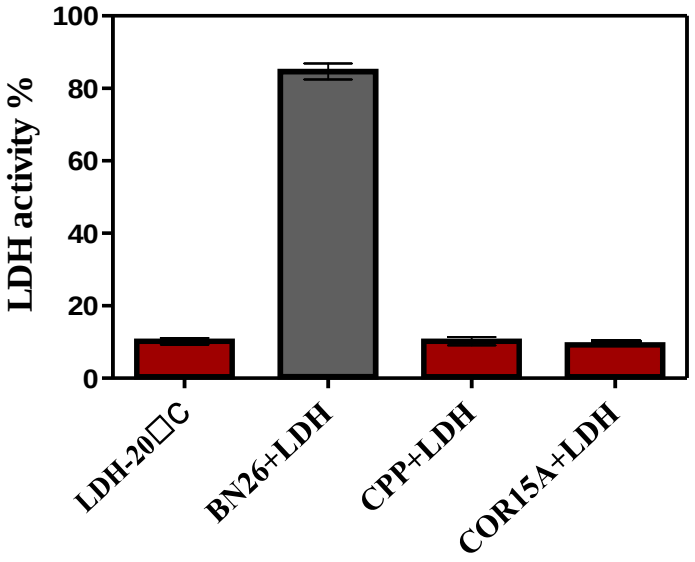


Figure 32: Recovery of lactate dehydrogenase activity by BN26. 5 µM BN26, CPP and COR15A suspended in10 mM potassium phosphate, pH 7.5 were frozen for 1 h and then thawed at room temperature. The proteins were added to freeze-denatured lactate dehydrogenase solution (1 µM), incubated for 30 min on ice and LDH activity was measured. Residual LDH activity was measured as percent of LDH activity before treatment (100%). Values are the means of (n=9) ± SD of 3 independent experiments.

4.4.10 Pull-down assay

In order to further unravel the mechanism by which BN26 protects the freeze-labile enzyme LDH against inactivation, we examined the ability of BN26 to associate with LDH *in vitro* by an immunopurification method. The pull-down assay is an *in vitro* method used to determine a physical interaction between two or more proteins.

Pull-down assays are useful for both confirming the existence of a protein-protein interaction predicted by other research techniques (e.g., co-immunoprecipitation) and as an initial screening assay for identifying previously unknown protein-protein interactions. As I know from my results that BN26 protects lactate dehydrogenase from different stresses *in vitro*, I set out to further analyze how BN26 protects enzymes from denaturation. From the previous results I had evidence that BN26 could maintain and recover lactate dehydrogenase activity. I used the pull-down assay in order to investigate the interaction between the BN26 and lactate dehydrogenase as a possible mechanism of cryoprotection. Freeze-stressed BN26, unstressed His-BN26, lactate dehydrogenase alone (-ve Protein) and *Pseudomonas syringae* allantoicase (ALC) his-tagged protein expressed in *E.coli* (Werner *et al.*, 2010) as a negative his-tag control were tested in this investigation.

Fig. 33 shows the pull-down assay revealing the interaction between stressed His-BN26 and LDH. The His-BN26 fusion protein was used as a bait to pull down the prey LDH with or without prior stress treatment of BN26 (Fig. 33). Stress treatment of BN26 was done via incubation of BN26 for 30 min at -20°C.

For a negative control, *Pseudomonas syringae* allantoicase (ALC) His-tagged protein expressed in *E.coli* (Werner *et al.*, 2010) was used. The upper panel shows the amount of bait and prey present in the prey loading step (FT), the wash steps one to three (W1-W3) and the elution of his-tagged protein (Elu) as revealed by a Coomassie Brilliant Blue-stained SDS-PAGE gel.

The different steps of the pull-down assay (FT, W1-W3 and Elu) were tested for relative LDH activity (lower panel). Only the freeze-stressed His-BN26 was able to interact with LDH. In the elution lane LDH co-eluted with prior stress treated His-BN26 (Fig. 33). In accordance this fraction (Elu) shows a high LDH activity (Fig. 33 lower panel). Unstressed BN26 was not able to interact with LDH (Fig. 33). Also no LDH activity could be observed in the fraction containing the eluted his tagged protein for unstressed BN26 (Fig. 33). Using another bait negative control His-tagged protein, *Pseudomonas syringae* allantoicase (ALC) was also not able to interact with LDH (Fig. 33). LDH alone does not bind to the beads and most LDH is found in the flow through (Fig. 33). We can conclude that BN26 as IDP becomes functional when stressed and therefore binds to its partner protein and prevents its inactivation.

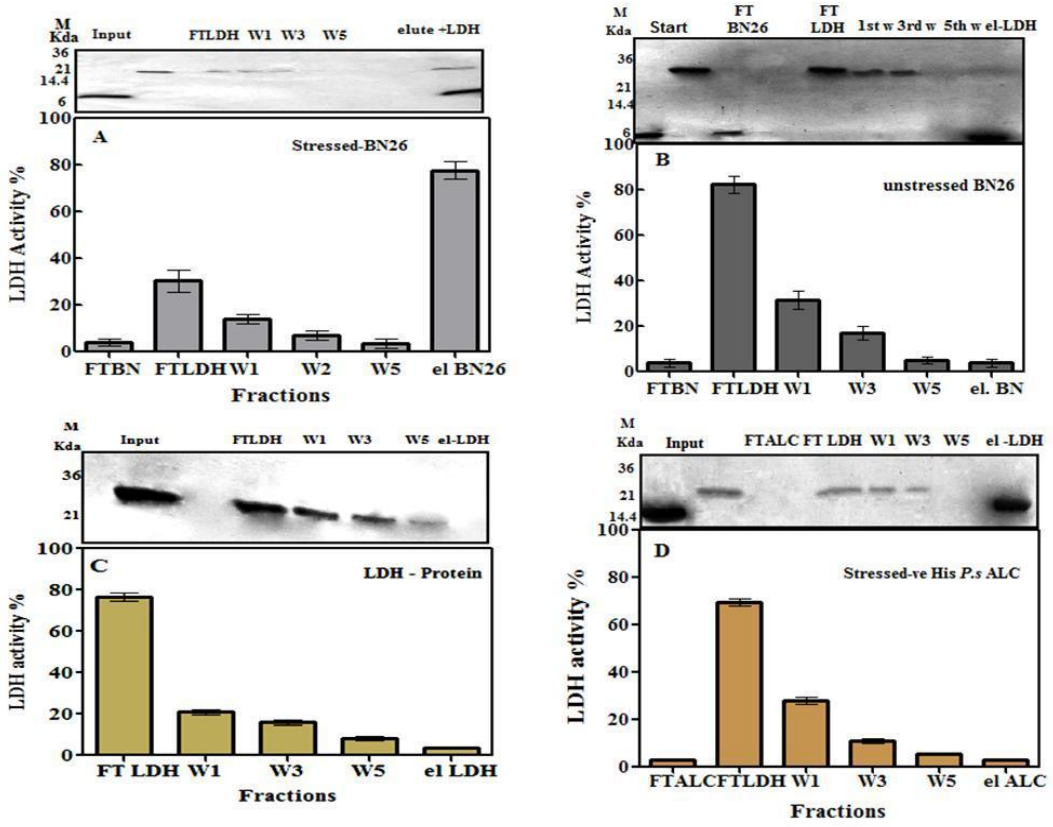


Figure 32: Pull-down asssay showing interaction between BN26 and Lactate dehydrogenase. As interaction partners for LDH stressed His-BN26 (A), unstressed His-BN26 (B) and *P. s.* allantoicase (ALC) His-tagged protein. (C) Were used and immobilized to 0.5 ml Ni-Sepharose 6 fast flow resin packed in microcolumns. Also LDH binding to Ni-Sepharose 6 was tested (D) The upper panel shows the amounts of bait and prey present in the bait or prey loading step (FT), the wash steps one to three (W1-W3) and the elution of his tagged protein (Elu) as revealed by a Coomassie Brilliant Blue-stained SDS-PAGE gel. BN26 and *P.s*ALC migrateat approximately 8,7 and 22 kDa, LDH at about 36 kDa, respectively. The lower panel shows LDH activity during the loading, washing and elution steps. Values represent the mean (*n*=9) % of prefrozen LDH of each fraction. LDH activity in the input was set to 100 %. The same LDH input was used independent from the tested interaction partner.

5.1 The role of COR and similar hydrophilic polypeptides in freezing tolerance

In the last two decades, hundreds of cold-regulated genes have been identified from a number of plant species like cabbage and spinach (Thomashow, 1994; Thomashow, 1998). A number of cold-regulated genes (*cor*, *Bn*, etc.) have been identified and characterized in several plants including *Arabidopsis thaliana*, *Brassica napus*, alfalfa, barley, wheat, tomato and other plants (Liu *et al.,* 2003). Studies have shown that the synthesis of certain COR proteins parallels the development of freezing tolerance (Houde *et al.*, 1995) and many *COR* genes are induced by drought (Kurkela *et al.*, 1988; Lin and Thomashow, 1992a; Nordin *et al.*, 1993). Proteins with known activities contributing to freezing tolerance are for example the *Arabidopsis* proteins COR6.6, COR15A, and COR78 polypeptides or homologues of LEA proteins such as *Arabidopsis* COR47 (Gilmour *et al.*, 1988; Thomashow, 1998). For *Brassica napus* low temperature regulated transcripts encoding for potential proteins, including BN26, could be identified (Orr *et al.*, 1997 ; Weretilnyk *et al.*, 1993). Results by Artus *et al.*, 1996 indicated a role for COR15A in freezing tolerance.

However, only a small number of polypeptides have been characterized at the molecular level and, therefore, the biochemical activities of cold-regulated proteins largely remain unclear. In this study, I have examined in detail the biochemical properties of a cold-regulated protein, BN26. *Bn26* has 69% homology with the amino acid sequence of *cor15a*. However, little is known about the structure and function of most of the low temperature inducible proteins in *Brassica napus.* For this reason, it will be interesting to compare the function of BN26 and COR15A and their biochemical behaviour. The aim of my thesis was to gain insights in the role of BN26 in freezing tolerance.

This was accomplished by applying a combination of various techniques which provide a detailed picture of the biochemical and biophysical properties of BN26. BN26 was expressed in *Pichia pastoris* and was purified via reverse phase chromatography (Fig.1 and Fig.2).

5.2 CD analysis and proteolytic sensitivity of BN26

5.2.1 Stress-induced structural changes in BN26

Most LEA proteins are predicted to be IDPs (intrinsically disordered proteins) in the fully hydrated state and may function as disordered proteins. Alternatively, they may acquire structure during partial or complete dehydration as brought about by freezing or drying (Hincha and Thalhammer, 2012). Owing to their hydrophilic unstructured nature, most LEA proteins do not aggregate during drying, freezing or even boiling, although there are some striking exceptions (Popova, *et al.*, 2011).

A search of a database of LEA protein sequences using the Fold Index unfolded-protein prediction tool (Prilusky *et al.*, 2005, Tunnacliffe, 2011) reveals that LEA proteins from groups 1, 2, 3 and the former group 4 are at least 50% unfolded. Lack of conventional secondary structure means that members of the major LEA protein groups are included in the large class of proteins variously called "natively unfolded", "intrinsically disordered" or "intrinsically unstructured" (Uversky *et al.*, 2000; Dunker *et al.*, 2001; Tompa 2002).

The first question addressed was if BN26 lacks a conventional secondary structure under unstressed conditions.

One of the features commonly associated with IDPs is their susceptibility to proteolysis due to their open and flexible conformational state (Uversky and Dunker, 2010). Due to the lack of a stable secondary structure, IDPs are generally more flexible than globular proteins (Otzen *et al.*, 2005). Comparisons of amino acid compositions of ordered proteins and IDPs revealed that IDPs are significantly depleted in aromatic amino acid residues (Trp, Tyr, Cys and Phe), which would normally form the hydrophobic core of a folded globular protein. Therefore, tryptophane, tyrosine, cysteine and phenylalanine were proposed to be called order-promoting amino acids (Dunker *et al.*, 2001; Romero *et al.*, 2001).

The depletion of IDPs in cysteine is also crucial, as this amino acid residue is known to have a significant contribution to the protein conformation stability (Romero *et al.*, 2001). For IDPs a high sensitivity to proteolytic digest could be shown (Tompa, 2002). Contrary, in globular domains cleavage sites are partially protected via secondary structure

elements thus globular proteins show significant higher resistance to proteolytic cleavage. This is because IDPs are usually very sensitive along their entire length, which results in much higher protease sensitivity, and this is a diagnostic for their open structures (Tompa, 2002). For BN26 a high sensitivity to proteolytic cleavage via trypsin could be shown (Fig. 21a) indicating a high amount of unstructured regions in BN26. The extreme proteolytic sensitivity of IDPs results from proteases cleaving substrates at sites that are accessible and flexible enough to make productive contacts with the protease. However, the globular protein lysozyme shows significant resistance and remains intact to proteolytic activity (Fig. 21b).

More in detail the secondary structure of BN26 was analyzed using CD spectroscopy. CD analysis revealed that BN26 is 65% unstructured with a minimal helical content (10%) in water (Fig. 19). However, upon drying the helical content increased to 50% and the unstructured content was reduced (20%) (Fig. 19). The high α-helix content of BN26 after dehydration suggests that the cryoprotection capacity of BN26 is accompanied by a structurally transition from random coil to amphipathic α-helix. This has also been previously suggested for COR15A by Lin *et al.* (1992).

Secondary structure prediction using different CD spectra analysis programs indicated that the BN26 homolog protein COR15A was approximately 70% unstructured in solution. Upon drying, the α-helical content of COR15A was about 65% (Thalhammer, 2010). So the observed structural transition from random coil to amphipathic α-helix shown via CD spectroscopy for BN26 reminds the described structural changes observed for COR15A.

Regarding the conservation of hydrophobic and hydrophilic amino acids between BN26 and COR15A a similar mechanism of structural changes seems probable. Even more the understanding of the relation between structural change and function in the case of the good described protein COR15A can lead to an idea of BN26 functionality.

5.3 BN26 Function

We suggest that BN26 functions as cryoprotectant. Two theories on cryoprotectant function were tested regarding BN26 functionality. The first theory predicts an enhancement of *membrane* stabilization by cryoprotectans (Santarius and Giersch, 1983). However, the other describes cryoprotectants that lead to *protein* stabilization (Carpenter *et al.*, 1988).

5.3.1 Can BN26 enhance membrane stability?

5.3.1.1 Can BN26 protect thylakoid membranes from freeze-induced damage?

In the last four decades, several investigators have reported that the leaves of cold-acclimated cabbage and spinach, but not non-acclimated plants, contain proteins that are effective in protecting isolated thylakoid membranes against freeze–thaw damage (Heber and Kempfle, 1970; Hincha *et al.*, 1989b). The effect of cryoprotective proteins in protection against freeze-thaw damage was established in detail for the plasma membrane (Steponkus, 1984) and chloroplast thylakoids (Hincha *et al.*, 1996). Isolated spinach thylakoids have been used as a model membrane system to determine whether additives can protect membranes against freezing injury (Hincha *et al.*, 1989; Hincha and Schmitt, 1992).

In my study I have used the *in vitro* assay established by Hincha and Schmitt (1992a, 1992b). After freeze-thaw cycles thylakoids from non-hardened plants rupture in the absence of cryoprotectant. In the presence of cryoprotectant thylakoids remain intact. The volumetric measurement distinguished between the ruptured one and the intact one.

BN26 can maintain 51% of the thylakoid integrity (Fig. 22). COR15A was able to maintain only 30% of the thylakoid membranes. In accordance with the capacity of BN26 to protect thylakoids from freeze-induced damage a strong binding of BN26 to the thylakoid membrane could be observed (Fig. 22). Sror *et al.*, 2003 showed that the protective effect of CPP is at least in part based on the binding of CPP to the thylakoid membrane. It was suggested that the appearance of an amphipathic α-helix is responsible

for the mechanism of CPP binding to thylakoid membranes. This is interesting regarding the stress induced structural change of BN26 leading to an increased helix content (see 1.2).

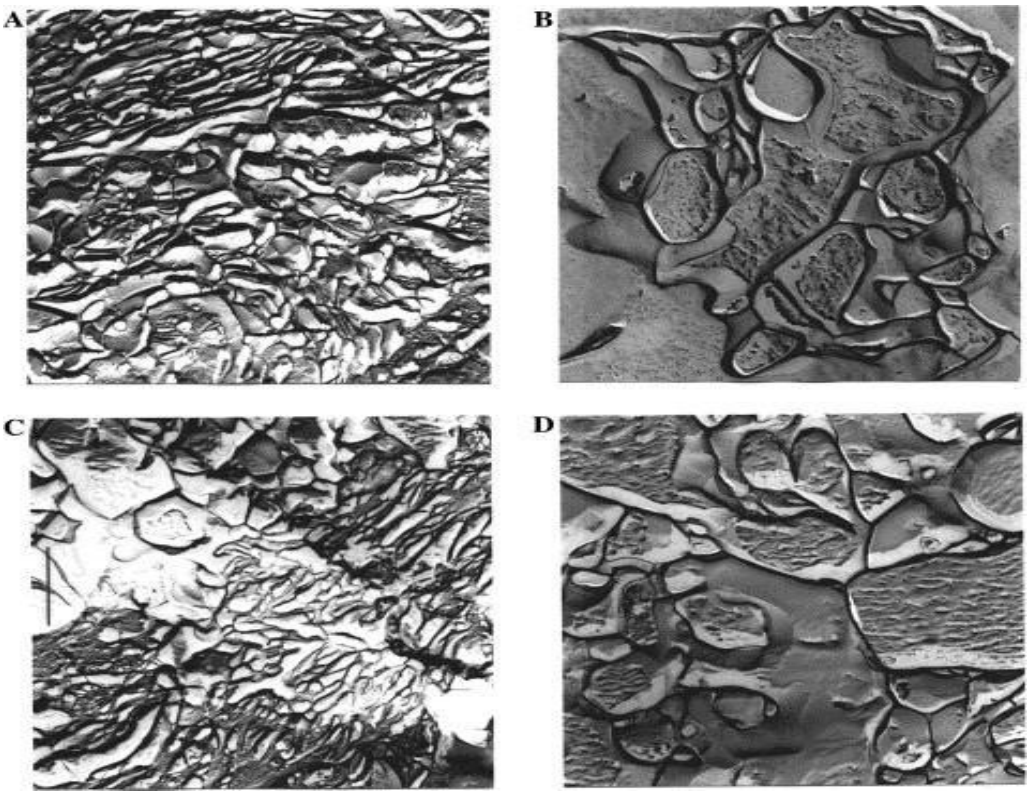


Figure 33: Freeze-fracture electron microscopy of thylakoisds from nonacclimated spinach leaves after a freeze–thaw cycle from Sror *et al.*, 2003. Membranes were incubated in the absence (A, C) or presence (B, D) of cryoprotectin and then frozen and thawed either directly (A, B), or after two (C, D) washing cycles. All electron microscopy pictures are shown at the same magnification. The bar in (C) represents 1 μm.

5.3.1.2 Does BN26 protect liposomes from freeze-induced dehydration damage?

The ability of BN26 to stabilize membranes was analyzed more in detail using liposomes. In contrast to a biological membrane, which also contains proteins, liposomes are only building of lipids thus enabling us to analyze the protection activity of BN26 for lipids bilayer. Liposomes mimicking the thylakoid membrane (Webb and Green, 1991) were tested. Peter Steponkus and colleagues (1993) used this method to determine whether the COR15am mature plastid-targeted polypeptide encoded by *cor15a* has any effects on the cryostability of liposomes *in vitro*. A number of intriguing observations have been made regarding the effects of COR15am on the cryostability and cryobehavior of liposomes (Webb *et al.*, 1996, Uemura *et al.*, 1996).

Liposomes were subjected to a dehydrative stress induced by freezing in the presence and absence of BN26. The carboxyfluorescein (CF) leakage from liposomes was measured as a sign of liposome integrity. My results show that BN26 moderately reduced liposome leakage by 20% (Fig. 27), suggesting that BN26 has no membrane function *in vivo*. In contrast, its homologue COR15A was able to prevent liposomes from freeze-induced leakage up to 60%. This result implicated that despite their similarity, different functions of BN26 and COR15A are probable. For COR15A for both, the thylakoid membrane and the lipid-build artificial thylakoid membranes, a function as cryoprotectant could be shown (see 1.3.1.1). Previously, it was hypothesized that COR15am can associate not only with freeze-labile stromal enzymes, but also with envelope membranes under certain conditions (Steponkus *et al.*, 1998).

Nakayama *et al.*, 2007 showed chloroplast stromal localization of COR15A. For BN26 chloroplast localization is predicted, suggesting that besides the lack of BN26 cryoprotection activity on liposomes a function of BN26 in thylakoids is probable. This is in accordance with the observed cryoprotection activity of BN26 for biological thylakoid membrane (see 1.3.1).

5.3.2 Can BN26 enhance protein stability and recover enzyme activity?

To further address the role of BN26 as a cryoprotectant, it was analyzed if BN26 is involved in the stabilization of proteins during freezing. In recent years, much insight has been gained about the basic mechanisms of protein stabilization under abiotic stress. The action of specific molecules, including chaperones, in protein stabilization was analyzed using several *in vitro* assays (Sun and Macrae, 2005; Basha *et al.*, 2006).

Previously, an *in vitro* freezing assay using lactate dehydrogenase (LDH) as the reporter enzyme had been described (Carpenter *et al.*, 1988; Lin and Thomashow 1992). This assay was used to test the protecting activity of different additives such as reducing sugars and importantly, LEA proteins from different groups and species (Tunnacliffe and Wise, 2007). Also, the assay was adapted to perform multiple freeze–thaw cycles or dehydration treatment to allow better visualization of the effect.

Such *in vitro* assays showed that some LEA proteins are able to stabilize sensitive enzymes during freezing and drying (Tunnacliffe and Wise, 2007; Hundertmark *et al.*, 2011)

One of these assays, to test the ability of compounds to prevent denaturation of the freeze-labile enzyme LDH, was performed following a freeze-thaw stress. This assay was previously used to determine the cyoprotective properties of COR15A (LEA-like protein) in *Arabidopsis thaliana* (Lin and Thomashow, 1992a).

COR15A was able to protect LDH from freeze-thaw damage better than other tested proteins such as BSA or RNAse by several orders of magnitude (Lin and Thomashow 1992a; Reyes *et al.*, 2005; Nakayama *et al.*, 2007; Nakayama *et al.*, 2008). As COR15A is a chloroplast-localized protein it is likely to protect chloroplasmatic enzymes from freeze- or chilling-induced inactivation.

Many overwintering plants contain cold-induced dehydrins, such as COR85 purified from cold-acclimated spinach leaves, WCS120 isolated from cold-acclimated winter wheat leaves, and PCA60 isolated from the bark of cold-acclimated peach trees which all provide cryoprotection of LDH activity at protein concentrations 10 to 100 times lower than BSA (Kazuoka and Oeda, 1994; Houde *et al.*, 1995). LEA proteins, most often

from group 2, can protect proteins such as LDH against freeze damage (Kazuoka and Oeda, 1994; Houde *et al.*, 1995; Wisniewsk *et al.*, 1999; Hara *et al.*, 2001; Sanchez-Ballesta *et al.*, 2004; Goyal *et al.*, 2005a). All main LEA protein groups seem able to offer protection to freeze sensitive enzymes such as LDH, malate dehydrogenase, fumarase and citrate synthase CS (Sanchez-Ballesta *et al.*, 2004; Reyes *et al.*, 2005). Goyal *et al.*, (2005a) have provided evidence that at least part of the protective function of LEA proteins is due to an ability to prevent aggregation of dehydration-sensitive proteins, Thereby these LEA proteins function as a molecular shield.

This is reminiscent of the role of molecular chaperones as stress protectants. However, chaperones not only prevent inappropriate protein aggregation but form specific, transient complexes with their client proteins, typically (although not exclusively) through interaction of hydrophobic patches (Tunnacliffe and Wise, 2007).

In the present study, BN26 affords virtually 96% protection from freeze-denaturation (Fig. 29). BN26 was shown to be a better cryoprotectant than COR15A, sucrose, CPP, and BSA. Moreover, it can retain 65% of LDH activity after three freeze-thaw cycles compared to the other tested additives (Fig. 30). Reyes *et al.*, (2005) reported that the cryoprotection offered by ERD10 and AtLEA4-5 is comparable after one and three freeze-thaw cycles.

Therefore, in this assay BN26 does appear to have cryoprotective properties. In addition BN26 also was able to conserve more than 60% of LDH activity after overnight dehydration (Fig. 31). However, dehydrated COR15A only conserve 35% of its initial activity. These data indicate that BN26 as COR15A has the ability to protect enzymatic activity from inactivation during different stresses *in vitro* as freezing and dehydration. Moreover, the addition of BN26 to a solution containing freeze treated LDH can recover LDH activity (Fig. 32). In contrast, neither COR15A nor CPP could recover the LDH enzyme activity (Fig. 32).

Figure 28 shows that BN26 was also able to recover luciferase activity up to 70%. However, COR15A and BSA were not able to recover luciferase activity. It was previously reported that COR15A has no chaperone activity, i.e. it is not able to refold proteins or assist in the refolding of denatured proteins (Nakayama *et al.*, 2008). The highly flexible and charged nature of these proteins (Kovacs *et al.*, 2008) explains the

action of IDP chaperones. However, chaperones not only prevent inappropriate protein aggregation but form specific, transient complexes with their client protein and entropic filling of space between client molecules (Fig. 36 b).

The ability of IDPs to bind to their target molecules, such as RNA, DNA and other (structured) proteins is of crucial importance for their function. In many cases it has been observed that binding induces increased secondary structure in IDPs (Tompa *et al.*, 2005) indicating their ability to fold under the appropriate conditions (Fig. 36). In the case of group 1 and group 3 LEA proteins, the evidence mostly points to their involvement in preventing aggregation of their target molecules caused by freezing (Wisniewsk *et al.*, 1999; Hara *et al.*, 2001; Momma *et al.*, 2003; Goyal *et al.*, 2005).

Based on these thoughts and regarding the LDH activity protecting function of BN26 direct binding of LDH and BN26 was analyzed using a pull-down assay (Fig. 33). In a first attempt no binding could be observed (Fig. 33B). For BN26 a stress induced increase of secondary structure elements could be observed (3.1). Therefore, I have used a prior stress treated BN26 as interaction partner in the pull down sassy. Under this condition an interaction with LDH could be observed (Fig. 33 A).

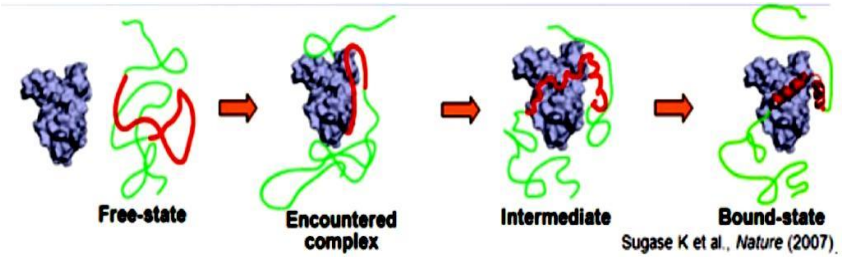


Figure 34: Schematic representaion shows IDPs. IDPs shown to have adopted structured regions upon binding to an interacting partner or another structured protein.

The functions of IDPs are usually intimately related to the structural characteristics of these proteins, such as high flexibility, structural adaptability, and extended conformational states. IDPs have been shown to have high ion-binding capacity (Heyen *et*

al., 2002 ; Alsheikh *et al.*, 2003; Tompa *et al.*, 2006), to be able to bind membranes (Daughdrill *et al.*, 1998; Ismail *et al.*, 1999a; Koag *et al.*, 2003), and to have both RNA and protein chaperone activity (Tompa and Csermely, 2004). The key elements of this mechanism are that disorder in chaperones may play a role in rather non-specific recognition, whereas it may also provide an effect of solubilization and assist local unfolding of misfolded parts of the client protein. Some of these functional elements are similar to "molecular shield" and "space filler" functions of LEA proteins (Fig 36b).

In conclusion, the stability during a freezing stress and the hydrophilic nature of the protein together with the secondary structure configuration of BN26 are all characteristics of a cryoprotective compound and strongly suggest a possible link between protein structure and function. BN26 resemble its homologue COR15A in enzyme stabilization ability in the case of stress treated LDH. This protection of LDH activity seems to result from the prevention of LDH aggregation during freezing similar to the function shown for other LEA_4 proteins (Prilusky *et al.*, 2005 ; Chakrabortee *et al.*, 2007).

But it could be shown, that contrary to COR15A, BN26 can enhance protein refolding of denaturated LDH or luciferase. Interestingly this function is ATP independent (Fig. 28).

These results suggest that BN26 might be related to "disordered chaperone-like" proteins by the three characteristics it has

1) Disordered chaperone- like proteins do not require an energy input in the form of ATP for functioning.

2) By functioning as molecular shields, disordered chaperone-like proteins can prevent aggregation of mis-folded proteins (Fig. 36).

3) Disordered chaperone-like proteins can enhance protein refolding of misfolded parts of the client protein.

Moreover, there is also a strong correlation between the observed function of BN26 and the ability of some IDPs to carry out multiple functions, termed moonlighting (Tompa, 2005). We can also conclude that COR15A and BN26 have both similar partially overlapping and special different *in vivo* functions. Artus *et al.*, 1996 previously mentioned that COR15A indeed has multiple protective activities, it may be possible to provide broad and robust protection of plant cells from freeze-induced damage simply by over expressing this single gene as mentioned. Of particular relevance to their stress-related functions is that for several IDPs and/or DHNs, chaperone or molecular shield function has already been demonstrated (Tompa and Csermely, 2004). Some important activities ascribed to ID proteins do not directly involve coupled binding and folding, but rather are dependent on the flexibility, pliability and plasticity of the backbone (Uversky *et al.*, 2000).

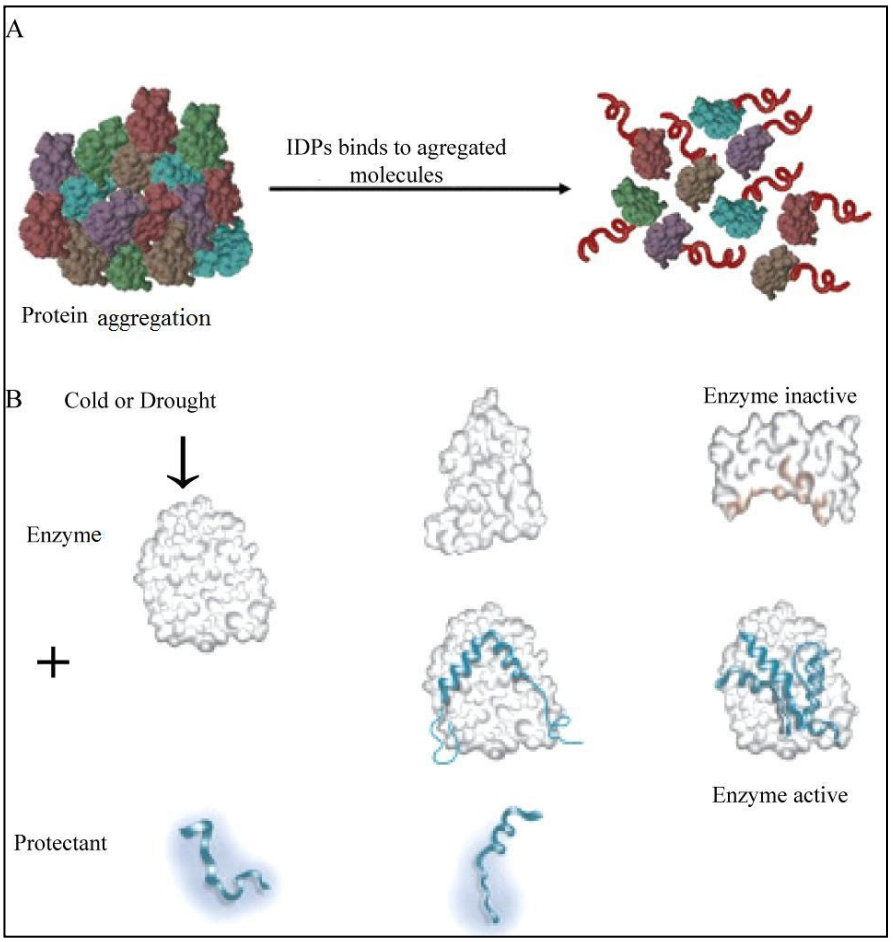


Figure 35: The suggested functions of a cryoprotectant in protein stabilization (A) It may form a physical barrier between neighboring partner molecules and preventing contact between them as a molecular shield from (Santner *et al.*, 2012). (B) Alternatively it may act as a chaperone to recover enzyme activity (Olvera-Carrillo *et al.*, 2011).

.

6 Summary

Cold acclimatization of plants is a multigenic trait during which multiple genes are expressed. One of the *Arabidopsis thaliana* cryoprotectant protein COR15A homologues in *Brassica napus* or *Brassica oleracea* with unknown function is BN26. The gene for *BN26* was cloned from *Brassica oleracea* and expressed in *Pichia pastoris* as a native protein without signal sequence and purified by reverse phase chromatography on Amberlite XAD7. A significant advantage offered by the use of the Amberlite XAD7 matrix is that the dried protein fractions can be stored without loss of activity for up to 4 to 6 months in the refrigerator and for one year in the freezer.

One main result obtained in this PhD thesis was the lack of secondary structural elements of BN26 in solution together with stress-induced folding of new secondary structure elements. Structural analysis via circular dichroism spectroscopy experiments revealed the folding of natively unstructured BN26 proteins during drying to mainly α-helical structure. Another diagnostic feature for unstructured proteins is their high proteolytic sensitivity. The higher protease sensitivity of unstructured proteins results from their open structure. Therefore, the polypeptide chain of unstructured proteins is more accessible to proteases than that of globular proteins. For BN26 sensitivity to proteolytic degradation could be shown, pointing to the flexible nature. In contrast, the globular protein lysozyme remains intact under the same conditions.

Further characterization in my thesis was done to enlighten the mode of action of BN26 protein. Thereby, both membrane and protein stabilization properties under stress were suggested as a possible mechanism of protection. BN26 protected thylakoids against freeze-thaw damage by binding to thylakoid membranes as shown by western blot analysis. Interestingly, BN26 shows in comparison to its high cryoprotecting ability for whole thylakoids just poor cryprotection ability for liposomes mimicking thylakoid membrane. Furthermore, BN26 has been shown to

exhibit *in vitro* cryoprotective activity on the freezing-labile enzyme, L-lactate dehydrogenase (LDH), against freeze-induced inactivation *in vitro*. The protection of proteins during drying on an LDH-assay could also be shown for BN26 protein.

In addition we examined the refolding activity of BN26 using firefly luciferase as a substrate. BN26 was found to stimulate folding of the molecule indicating a chaperone activity of BN26. In conclusion our data indicate that BN26 functions as protectant against various stresses.

Furthermore I could show in pull-down assays that only stress treated BN26 but not unstressed BN26 can bind to LDH. This is interesting regarding the fact that secondary structure elements in BN26 are formed via stress treatment. I suggest that the structural changes of BN26 are necessary for binding to potential targets and even more for its function as protectant against various stresses.

Zusammenfassung

Bei der Kälteakklimatisation von Pflanzen spielen eine Vielzahl von Genen eine Rolle. Diese Arbeit untersucht das bis dahin funktionell nicht beschriebene Protein BN26. Das *Brassica napus* und *Brassica oleracea* Protein BN26 ist homolog zu dem *Arabidopsis* Frostschutz Protein COR15A. Die kodierende Sequenz von *BN26* wurde aus *Brassica oleracea* isoliert. Die Expression erfolgte als natives Protein ohne Signalsequenz in *Pichia pastoris*. Die anschließende Aufreinigung erfolgte mittels Umkehrphasenchromatographie unter Verwendung einer Amberlite XAD7 Matrix. Ein wesentlicher Vorteil bei der Verwendung der Amberlite XAD7 Matrix ist, dass die getrockneten Proteinfraktionen ohne Aktivitätsverlust 4 bis 6 Monate im Kühlschrank und ein Jahr im Gefrierschrank gelagert werden können.

Ein Hauptresultat dieser Arbeit ist das Fehlen von Sekundärstrukturelementen im gelösten BN26 und die stressabhängige Ausbildung neuer Sekundärstrukturen. In der Charakterisierung der BN26 Sekundärstruktur mittels Zirkulardichroismus-Spektroskopie konnte ein hoher Anteil an ungefalteten Proteinregionen im gelösten BN26 und eine dem Wasserentzug folgende deutliche Zunahme an α-Helices beobachtet werden. Ein anderes Verfahren zum Nachweis von Proteinen mit einer großen Anzahl an unstrukturierten Bereichen in der Sekundärstruktur macht sich die Empfindlichkeit solcher unstrukturierten Proteinbereiche gegenüber Proteasen zunutze. Die höhere Protease-Empfindlichkeit von unstrukturierten Proteinen ergibt sich aus ihrer offenen Struktur. Die Protease kann an die Polypeptidkette von unstrukturierten Proteinen deutlich besser binden als an die von globulären Proteinen. Es gelang für gelöstes BN26 eine hohe Proteasesensitivität nachzuweisen, was auf einen hohen Anteil an unstrukturierten Proteinregionen hindeutet. Im Gegensatz dazu war unter den gleichen experimentellen Bedingungen für das globuläre Proteins Lysozym ein deutlich höherer Anteil des gesamten Proteins intakt und unverdaut.

Eine weitere Charakterisierung von BN26 im Rahmen meiner Doktorarbeit diente der funktionellen Untersuchung. Als mögliche Mechanismen des abiotischen Stressschutzes wurden die Stabilisation von Membranen und Proteinen diskutiert. Für BN26 konnte eine Funktion beim Schutz von Thylakoiden gegen Frost-Tau-Schäden und eine Bindung an Thylakoidmembranen durch Western-Blot-Analyse gezeigt werden. Interessanterweise konnte im Gegensatz zum Frostschutz von Gesamt-Thylakoiden nur ein geringer Frostschutz von Liposomen, die als Modell für Thylakoidmembranen verwendet wurden, durch BN26 beobachtet werden. Des Weiteren schützte BN26 das kälteempfindliche Enzym L-Lactatdehydrogenase (LDH) beim Einfrieren *in vitro* vor einem Aktivitätsverlust. Auch bei Wasserentzug durch Trocknen konnte eine schützende Funktion von BN26 für LDH gezeigt werden.

Zudem konnte für das Substrat Luciferase gezeigt werden, dass BN26 die Faltung von Proteinen stimuliert, was auf eine Funktion als Chaperon hinweist. Die Daten zeigen eine Funktion von BN26 als abiotisches Stress-Schutzprotein auf.

Außerdem konnte ich in Pull-Down Untersuchungen zeigen, dass nur Stress-behandeltes BN26 Protein, aber nicht "ungestresstes" BN26 LDH binden kann. Das ist interessant im Hinblick auf die Tatsache, dass durch Stress-Behandlung sekundäre Strukturelemente in BN26 gebildet werden. Ich schlage vor, dass die strukturellen Veränderungen in BN26 notwendig für die Bindung an potenzielle Ziele und noch mehr für seine Funktion als Schutzmittel gegen verschiedene Stressfaktoren sind.

Al-Shehbaz, I.A., Beilstein, M.A., and Kellogg, E.A. (2006). Systematics and phylogeny of the Brassicaceae (Cruciferae): An overview.*Plant Syst Evol.* **259**: 89–120.

Alsheikh, M.K., Heyen, B.J., and Randall, S.K. (2003). Ion binding properties of the dehydrin ERD14 are dependent upon phosphorylation. *J Biol. Chem.* **278**: 40882–40889.

Arnon, D. (1949). Copper Enzymes in Isolated Chloroplasts. Polyphenoloxidase in *Beta Vulgaris*. *Plant Physiol.* **24**: 1-15.

Artus, N.N., Uemura, M., Steponkus, P.L., Gilmour, S.J., Lin, C., and Thomashow, M.F. (1996). Constitutive expression of the cold-regulated *Arabidopsis thaliana* COR15a gene affects both chloroplast and protoplast freezing tolerance. *Proc. Natl. Acad. Sci. U. S. A* **93**: 13404–13409.

Azocar, A, Rada, F., and Goldstein, G. (1988). Freezing tolerance in *Draba chionophila*, a miniature caulescent rosette species. *Oecologia* **75**: 156–160.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5'-region of *Arabidopsis thaliana cor15a* has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. *Plant Mol. Biol.* **24**: 701–713.

Bangham, A.D., (1972). Lipid Bilayers and Biomembranes. *Ann. Review of Biochem.* **41**: 753-776.

Basha, E., Friedrich K.L., and Vierling, E. (2006). The N-terminal arm of small heat shock proteins is important for both chaperone activity and substrate specificity. *J Biol Chem* **281**: 39943–39952.

Beilstein, M.A., Al-Shehbaz, I.A. and Kellogg, E.A. (2006). Brassicaceae phylogeny and trichome evolution. *Ann J. Bot.* **93**: 607–619.

Boyer, J.S. (1982). Plant productivity and environment. *Science* **218**: 443-448.

Bradford, M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* **72**: 248-254.

Bray, E.A., Bailey-Serres, J., and Weretilnyk, E. (2000). Responses to abiotic stresses. *In*: W. Gruissem, B. Buchannan, R. Jones, eds, Biochemistry and Molecular Biology of Plants 1158–1249.

Browse, J., and Xin, Z. (2001). Temperature sensing and cold acclimation. *Curr. Opin. Plant Biol.* **4**: 241-246.

Buhot, N., Gomes, E., Milat, M., Ponchet, M., Marion, D., Lequeu, J., Delrot, S., Coutos-Thevenot, P. and Blein, J. (2004). Modulation of the biological activity of a tobacco LTP1 by lipid complexation. *Mol Biol Cell* **15**: 5047-5052.

Burke, M.J., Gusta, L.V., Quamme, H.A., Weiser, C.J., and Li, P.H. (1976). Freezing and Injury in Plants. *Ann. Rev. Plant Physiol.* **27**: 507-528.

Cabrita, L.D. and Bottomley, S.P. (2004). Protein expression and refolding–A practical guide to getting the most out of inclusion bodies. *Biotechnology Annual Review*, Elsevier. **10:** 31-50.

Carpenter, J.F., and Crowe, J.H. (1988). The mechanism of cryprotection of proteins by solutes. *Cryobiology* **25**: 244-255.

Cereghino, J.L. and Cregg, J.M. (2000). Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. *FEMS Microbiol Rev.* **24**: 45–66.

Chakrabortee, S., Boschetti C., Walton L.J., Sarkar S., Rubinsztein, D.C. and Tunnacliffe, A. (2007). Hydrophilic protein associated with desiccation tolerance exhibits broad protein stabilization function. *Proc Natl. Acad. Sci. U.S.A* **104**: 18073-18078.

Chen, T.H.H. (1994). Plant adaptation to low temperature stress. *Can. J. Plant Pathol.* **16**: 231-236.

Cregg, J.M., Tolstorukov, I., Kusari, A., Sunga, J., Madden, K., and Chappell, T. (2009). Expression in the yeast *Pichia pastoris*. *Methods Enzymol*. **463**: 169-189.

Daly, R. and Hearn, M.T. (2005). Expression of heterologous proteins in *Pichia pastoris*: A useful experimental tool in protein engineering and production. *J Mol. Recognit.* **18**: 119-138.

Daughdrill, G.W., Hanely L.J., and Dahlquist, F.W. (1998). The C-terminal half of the anti-sigma factor FlgM contains a dynamic equilibrium solution structure favoring helical conformations. *Biochem*. **37**: 1067-1082.

De Schutter, K.Y., Lin, P., Tiels, A., Van Hecke, S., Glinka, J., Weber-Lehmann, P., Rouzé, Y., Van de Peer and Callewaert, N. (2009). Genome sequence of the recombinant protein production host *Pichia pastoris*. *Nature Biotechnol.* **27**: 561-566.

Dunker, A.K., Lawson, J.D., Brown, C.J., Williams, R.M., Romero, P., Oh, J.S., Oldfield, C.J., Campen, A.M., Ratliff, C.M., Hipps K.W., Ausio, J., Nissen M.S., Reeves, R., Kang C., Kissinger C.R., Bailey R.W., Griswold, M.D., Chiu, W., Garner, E.C. Obradovic, Z. (2001). Intrinsically disordered protein. *J Mol Graph Model* **19**: 26–59.

Ellis, R.J. (2004). From chloroplasts to chaperones: How one thing led to another. *Photosynth Res.* **80**: 333–343.

Fowler, S. and Thomashow, M.F. (2002). *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. *Plant Cell* **14**: 1675-1690.

Gilmour, S.J., Hajela, R.K. and Thomashow, M.F. (1988). Cold acclimation in *Arabidopsis thaliana*. *Plant Physiol.* **87**: 745-750.

Gilmour, S.J., Lin, C. and Thomashow, M.F. (1996). Purification and properties of *Arabidopsis thaliana* COR (Cold-Regulated) gene polypeptides COR I5am and COR6.6 expressed in *Escherichia coli*. *Plant Physiol.* **111**: 293-299.

Gordon-Kamm, W.J. and Steponkus, P.L. (1984). Lamellar- tohexagonal11 phase transitions in the plasma membrane of isolated protoplasts after freeze-induced dehydration. *Proc Natl. Acad. Sci. U. S .A* **81**: 6373-6377.

Goyal, K., Walton, L.J. and Tunnacliffe, A. (2005). LEA proteins prevent protein aggregation due to water stress. *Biochem. J.* **388**: 151–157.

Griffith, M., Lumb, C., Wiseman, S.B., Wisniewski, M., Johnson, R.W., and Marangoni, A.G. (2005). Antifreeze proteins modify the freezing process in planta. *Plant Physiol.* **138**: 330-340.

Guy, C.L. (1990). Cold acclimation and freezing stress tolerance: role of protein metabolism. *Ann Rev Plant Physiol Plant Mol. Biol.* **41**: 187-223.

Greenfield, N.J. (1996). Methods to estimate the conformation of proteins and polypeptides from circular dichroism data. *Anal. Biochem.* **235**: 1–10.

Hara, M., Terashima, S. and Kuboi, T. (2001). Characterization and cryoprotective activity of cold-responsive dehydrin from *Citrus unshiu*. *J Plant Physiol.* **158**: 1333-1339.

Hays, L.M., Feeney, R.E., Crowe, L.M., Crowe, J.H. and Oliver, A.E. (1996). Antifreeze glycoproteins inhibit leakage from liposomes during thermotropic phase transitions. *Proc Natl. Acad. Sci. U.S.A* **93**: 6835-6840.

Heber, U. and Kempfle, M. (1970). Proteine als Schutzstoffe gegenüber dem Gefriertod der Zelle. *Z. Naturforsch.* **25b**: 834-842.

Heino, P. and Palva, E.T. (2003). Signal transduction in plant cold acclimation. *In: Hirt H, Shinozaki K (eds) Plant Responses to Abiotic Stress. Topics in Current Genetics. Springer- Verlag*, Berlin Heidelberg: 151–186.

Hennessey, J.R., J.P., J., Johnson, J.R. and W.C. (1981). Information content in the circular dichroism of proteins. *Biochemistry* **20**: 1085–1094.

Heyen, B.J., Alsheikh, M.K., Smith, E.A., Torvik, C.F., Seals, D.F. and Randall, S.K. (2002). The calcium-binding activity of a vacuole-

associated,dehydrin-like protein is regulated by phosphorylation. *Plant Physiol.* **130**: 675–687.

Higgins, D., Busser, K., Comiskey, J., Whittier, P., Purcell, T. and Hoeffler, J. (1998a). Small Vectors for Expression Based on Dominant Drug Resistance with Direct Multicopy Selection. *Pichia Protocols*. D. Higgins and J. Cregg, Humana Press. **103:** 41-53.

Higgins, D. and Cregg, J. (1998b). *Pichia* protocols: Methods in Molecular Biology. *Humana Press Totowa* New Jersey **103**.

Hincha, D.K., Heber, U. and Schmitt, J.M. (1989b). Freezing ruptures thylakoid membranes in leaves and rupture can be in prevented *in vitro* by cryoprotective proteins. *Plant Physiol. Biochem.* **27**: 795-801.

Hincha, D.K., Heber, U. and Schmitt, J.M. (1990). Proteins from frost-hardy leaves protect thylakoids against mechanical freeze-thaw damage *in vitro*. *Planta* **180**: 416-419.

Hincha, D.K. and Schmitt, J.M. (1992). Freeze-thaw injury and cryoprotection of thylakoid membranes. In: Water and Life: Comparative analysis of water relationships at the organismic, cellular, and molecular levels. G.N. Somero, C.B. Osmond, and C.L. Bolis, eds., Springer, pp 316-337.

Hincha, D.K. and Schmitt, J.M. (1992a). Cryoprotective leaf proteins: assay methods and heat stability. *J. Plant Physiol.* **140**: 236-240.

Hincha, D.K., Sieg, F., Bakaltcheva, I., Köth, H. and Schmitt, J.M. (1996). Freeze-Thaw damage to thylakoid membranes: specific protection by sugars and proteins. *In*: Advances in low-Temperatures Biology. Steponkus, P.L. (ed.). *JAI Press, London* **3**: 141-183.

Hincha, D.K. (2008). Effects of α-tocopherol (vitamin E) on the stability and lipid dynamics of model membranes mimicking the lipid composition of plant chloroplast membranes. *FEBS Letters* **582**: 3687-3692.

Hincha, D.K. and Thalhammer, A. (2012). LEA proteins: IDPs with versatile functions in cellular dehydration tolerance. *Biochem Soc. Trans.* **40**: 1000-1003.

Horvath, I., Glatz, A., and Varvasovszki, V. (1998). Membrane physical state controls the signaling mechanism of the heat shock response in *Synechocystis* PCC 6803: identification of *hsp17* as a Fluidity gene. *Proc Natl. Acad. Sci. U.S.A* **95**: 3513-3518.

Houde, M., Daniel, C.L., Achapelle, M., Allard, F., Laliberte, S. and Sarhan, F. (1995). Immunolocalization of freezing-tolerance-associated proteins in the cytoplasm and nucleoplasrn of wheat crown tissues. *Plant J.* **8**: 583-593.

Hundertmark, M., Dimova, R., Lengefeld, J., Seckler, R. and Hincha, D. K. (2011). The intrinsically disordered late embryogenesis abundant protein LEA18 from *Arabidopsis thaliana* modulates membrane stability through

binding and folding. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1808**: 446-453.

Ismail, A., Hall, A.E. and Close, T.J. (1999a). Allelic variation of a dehydrin gene cosegregates with chilling tolerance during seedling emergence. *Proc Natl Acad. Sci. U.S.A.* **96**: 13566–13570.

Jan, N.U., Hussain, M. and Andrabi, K. (2009). Cold resistance in plants: A mystery unresolved. *Electronic Journal of Biotechnology* **12**.

Jones, H.G. and Jones, M.B. (1989). Introduction: some terminology and common mechanisms. H.G. Jones, T.J. Flowers, M.B. Jones (*Eds.*), Plants Under Stress, Cambridge university Press, Cambridge: 1–10.

Kacperska, A. (1989). Metabolic consequences of low temperature stress in chilling-insensitive plants. *In*: PH. Li, ed. Low Temperature Stress Physiology in Crops. CRC Press, Boca Raton, Fl. :27–40.

Kazuoka, T. and Oeda, K. (1994). Purification and characterization of COR85-oligomeric complex from cold-acclimated *spinach*. *Plant Cell Physiol.* **35**: 601–611.

Kenefick, D. (1963). *Crop Science* **3**: 202–205.

Kis-Papo, T., Kirzhner, V., Wasser, S.P. and Nevo, E. (2003). Evolution of genomic diversity and sex at extreme environments: fungal life under hypersaline Dead Sea stress. *Proc.Natl. Acad. Sci. U.S.A.* **100**: 14970–14975.

Knight, H. and Knight, M.R. (2000). Imaging spatial and cellular characteristics of low temperature calcium signature after cold acclimation in *Arabidopsis*. *Experimental Botany* **51**: 1679–1686.

Knight, H., and Knight, M.R. (2001). Abiotic stress signalling pathways:specificity and cross-talk. *Trends Plant Sci.* **6**: 262– 267.

Knight, H., Trewavas, A.J. and Knight, M.R. (1996). Cold calcium signaling in *Arabidopsis* involves two cellular pools and a change in calcium signature after acclimation. *The Plant Cell* **8**: 489–503.

Knight, H., Zarka, D.G., Okamoto, H., Thomashow, M.F. and Knight, M.R. (2004). Abscisic acid induces CBF gene transcription and subsequent induction of cold-regulated genes via the CRT promoter element. *Plant Physiol.* **135**: 1710–1717.

Knight, M.R., Campbell, A.K., Smith, S.M. and Trewavas, A.J. (1991). Transgenic plant aequorin reports the effects of touch and cold-shock and elicitors on cytoplasmic calcium. *Nature* **352**: 524–526.

Knight, M.R. and Knight, H. (2012). Low-temperature perception leading to gene expression and cold tolerance in higher plants. *New Phytologist* **195**: 737–751.

Koag, M.C., Fenton, R.D., Wilkens, S. and Close, T.J. (2003). The binding of maize DHN1 to lipid vesicles. Gain of structure and lipid specificity *Plant Physiol* **131**: 309–316.

Koch, M. (2001). Molecular systematics of the Brassicaceae: evidence from plastidic *matK* and *Chs* sequences. *Am J. Bot.* **88**: 534–544.

Koch, M., Haubold, B. and Mitchell-Olds, T. (2000). Comparative evolutionary analysis of chalcone synthase and alcohol dehydrogenase loci in *Arabidopsis*, *Arabis* and related genera (Brassicaceae). *Mol. Biol. Evol.* **17**: 1483–1498.

Koch, M.A. and Al-Shehbaz, I.A. (2002). Molecular data indicate complex intra- and intercontinental differentiation of American Draba (Brassicaceae). *Ann. Missouri Bot. Gard.* **89**: 88–109.

Kovacs, D., Agoston, B. and Tompa, P. (2008). Disordered plant LEA proteins as molecular chaperones. Plant Signaling & Behavior **3**: 710-713.

Kreps, J.A. Wu, Y. Chang, H.S., Zhu, T. and Wang, X. (2002). Transcriptome changes for *Arabidopsis* in response to salt, osmotic, and cold stress. *Plant Physiol.* **130**: 2129–2141.

Kurkela, S. and Franck, M. (1990). Cloning and characterization of a cold-and ABA-inducible *Arabidopsis* gene. Plant Molecular Biology **15**1: 137-144.

Kurkela. S., Franck, M., Heino, P., Ling, V. and Palva, E.T. (1988). Cold induced gene expression in *Arabidopsis thaliana* (L). *Plant Cell Reports* **7**: 495-498.

Lång, V., Heino, P. and Palva, E.T. (1989). Low-temperature acclimation and treatment with exogenous abscisic-acid induce common polypeptides in *Arabidopsis thaliana* (L). *Heynh. Theor Appl. Genet.* **77**: 729-734.

Lee, G.J. and Vierling, E. (2000). A small heat shock protein cooperates with heat shock protein 70 systems to reactivate a heat-denatured protein. *Plant Physiol.***122**: 189–198.

Lee, S.P., Zhu, B., Chen, T.H.H. and Li, P.H. (1992). Induction of freezing tolerance in potato (*Solanum commersonü*) suspension cultured cells. *Physiologia Plantarum* **84**: 41–48.

Levitt, J. (1980). Responses of plants to environmental stresses. I: Chilling, freezing and high temperature stresses. *Academic Press, Orlando*.

Lin-Cereghino, Hashimoto, J., Moy, M.D., Castelo, A., Orazem, J., Kuo, C.C., Xiong, P., Gandhi, S., Hatae, V., Chan, C.T. and Lin-Cereghino, G.P. (2008). Direct selection of *Pichia pastoris* expression strains using new G418 resistance vectors. *Yeast* **25**: 293–299.

Lin, C. and Thomashow, M.F. (l992a). A cold-regulated *Arabidopsis* gene encodes a polypeptide having potent cryoprotective activity. *Biochem. and Biophys. Research Comm.* **183**: 1103-1108.

Lin, C. and Thomashow, M.F. (1992b). DNA sequence analysis of a complementary DNA for cold-regulated *Arabidopsis* gene *cor15* and characterization of the COR15 polypeptide. *Plant Physiol.* **99**: 519-525.

Lynch, D.V. and Steponkus, P.L. (1987). lasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. Puma). *Plant Physiol.* **83**: 761-767.

Mahajan, S. and Tuteja, N. (2005). Cold, salinity and drought stresses: An overview. *Archives of Biochemistry and Biophysics* **444**: 139-158.

Martin, R., Glover-Cutter, K., Baldwin, J. and Dombrowski, J. (2012). Identification and characterization of a salt stress-inducible zinc finger protein from Festuca arundinacea. *BMC Research Notes* **5**: 66.

Martinieire, A., Shvedunova, M., Thomson, A.J., Evans, N.H., Penfield, S., Runions, J. and McWatters, H.G. (2011). Homeostasis of plasma membrane viscosity in fluctuating temperatures. *New Phytologist* **192**: 328–337.

Maximov, N.A. (1912). Chemische schutzmittel der pflanzen gegen erfrieren. *Ber. Deutsch. Bot. Ges.* 30: 52.

Mohapatra, S.S., Poole, R.J. and Dhindsa, R.S. (1987). Changes in Protein Patterns and Translatable Messenger RNA Populations during Cold Acclimation of Alfalfa. *Plant Physiol.* **84**: 1172-1176.

Momma, M., Kaneko S., Haraguchi, K. and Matsukura, U. (2003). Peptide mapping and assessment of cryoprotective activity of 26/27-kDa dehydrin from soybean seeds. *Biosci Biotechnol. Biochem.* **67**: 1832–1835.

Monroy, A.F. and Dhindsa, R.S. (1995). Low temperature signal transduction: induction of cold acclimation–specific genes of alfalfa by calcium at 25 ºC. *Plant Cell Environ.* 7: 321-331.

Nakayama, K., Okawa, K., Kakizaki, T., Honma, T., Itoh, H. and Inaba, T. (2007). *Arabidopsis* Cor15am is a chloroplast stromal protein that has cryoprotective activity and forms oligomers. *Plant Physiol.* **144**: 513-523.

Nakayama, K., Okawa, K., Kakizaki, T. and Inaba, T. (2008). Evaluation of the Protective Activities of a Late Embryogenesis Abundant (LEA) Related Protein, Cor15am, during Various Stresses *in Vitro*. *Bioscience, Biotechnology, and Biochemistry* **72**: 1642-1645.

Neven, L.G., Haskell, D.W., Hofig, A., Li, Q.-B.and Guy, C.L. (1993). Characterization of a spinach gene responsive to low temperature and water stress. *Plant Mol. Biot.* **21**: 291-305.

Nevo, E. (2001). Evolution of genome-phenome diversity under environmental stress. *Proc Natl. Acad Sci. U S A* **98**: 6233–6240.

Nielsen, R. (2005). Molecular signatures of natural selection. *Ann. Rev. Genet.* **39**: 197–218.

Nordin, K., Vahala, T. and Palva, E.T. (1993). Differential expression of two related, lowtemperature-induced genes in *Arabidopsis thaliana* (L.) Heynh. *Plant Mol. Biol.* **21**: 641-653.

Ohno, S. (1970). Evolution by GeneDuplication. *Springer-Verlag Press,Heidelberg, Germany*.

Oliver, J.A., Monroe, D.M., Church, F.C., Roberts, H.R. and Hoffman, M. (2002). Activated protein C cleaves factor Va more efficiently on endothelium than on platelet surfaces. *Blood* **100**: 539-546.

Olvera-Carrillo, Y., Luis Reyes, J. and Covarrubias, A.A. (2011). Late embryogenesis abundant proteins: Versatile players in the plant adaptation to water limiting environments. *Plant Signaling & Behavior* **6**: 586-589.

Orr, W., lu, B., White, T.C., Robert. L.S. and Singh, J. (1992). Complementary DNA sequence of a low temperature-induced *Brassica napus* gene with homology to the *Arabidopsis thaliana* gene. *Plant Physiol.* **98**: 1532- 1534.

Örvar, B.L., Sangwan, V., Omann, F. and Dhindsa, R.S. (2000). Early steps in cold sensing by plant cells: the role of actin cytoskeleton and membrane fluidity. *Plant Journal* **23**: 785-794.

Palta, J.P. (1989). Plasma membrane ATPase as a key site of perturbation in response to freeze-thaw stress. *Blevins, eds, Current Topics in Plant Biochemistry and Physiology* **8**: 41-68.

Pearce, R.S. (1999). Molecular analysis of acclimation to cold. *Plant Growth Regulation* **29**: 47-76.

Pearce, R.S. (2001). Plant Freezing and Damage. *Annuals of Botany* **87**: 417-424.

Perras, M. and Sarhan, F. (1989). Synthesis of freezing tolerance proteins in leaves, crown, and mots during cold acclimation of wheat. *Plant Physiol.* **89**: 577-585.

Popova, A.V., Hundertmark, M., Seckler, R. and Hincha, D.K. (2011). Structural transitions in the intrinsically disordered plant dehydration stress protein LEA7 upon drying are modulated by the presence of membranes. *Biochimica et Biophysica Acta (BBA)-Biomembranes* **1808**: 1879-1887.

Prilusky J., Felder C.E., Zeev-Ben-Mordehai, T., Rydberg, E.H., Man O., Beckmann, J.S., Silman I. and J.L., S. (2005). FoldIndex: a simpletool to predict whether a given protein sequence is intrinsically unfolded. *Bioinformatics* **21**: 3435–3438.

Reyes, J.L., Rodrigo, M.J., Colmenero-Flores, J.M., Gil, J.V., Garay-Arroyo, A. and Campos, F. (2005). Hydrophilins from distant organisms can protect enzymatic activities from water limitation effects *in vitro*. *Plant Cell and Environment* **28**: 709-718.

Romanos, M. (1995). Advances in the Use of *Pichia pastoris* for High-Level Expression. *Curr. Opin. Biotech.* **6**: 527-533.

Rosas, A., Alberdi, M., Delseny, M. and Luis, M.-B. (1986). A cryoprotective polypeptide isolated from Nothofagus dombeyi seedlings. *Phytochemistry* **25**: 2497-2500.

Rubinsky, B., Arav, A., and DeVries, A.L. (1991a). Cryopreservation of oocytes using directional cooling and antifreeze glycoproteins. *Cryo Lett.* **12**: 93-106.

Sambrook, J. and Russell, D.W. (2001). *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press.

Sanchez-Ballesta, M.T., Rodrigo, M.J., Lafuente, M.T., Granell, A. and Zacarias, L. (2004). Dehydrin from Citrus, which confers *in vitro* dehydration and freezing protection activity, is constitutive and highly expressed in the flavedo of fruit but responsive to cold and water stress in leaves. *J. Agric. Food Chem.* **52**: 1950–1957.

Santarius, K.A. and Giersch, C. (1983). Cryopreservation of spinach chloroplast membranes by low-molecular-weight carbohydrates.II .Discrimination between colligative and non-colligative protection. *Cryobiology* **20**: 90-99.

Santner, A.A., Croy, C.H., Vasanwala, F.H., Uversky, V.N., Van, Y.-Y. J. and Dunker, A.K. (2012). Sweeping Away Protein Aggregation with Entropic Bristles: Intrinsically Disordered Protein Fusions Enhance Soluble Expression. *Biochemistry* **51**: 7250-7262.

Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **166**: 368-379.

Seki, M., Narusaka, M., Abe, H., Kasuga, M., Yamaguchi-Sjinozaki, K., Carinici, P., Hayashizaki, Y. and Shinpzaki, K. (2001). Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. *Plant Cell Environ.* **13**: 61-72.

Sharma, P., Sharma, N. and Deswal, R. (2005). The molecular biology of the low-temperature response in plants. *Bioessays* **27**: 1048–1059.

Shinozaki, K. and Yamaguchi-Shinozaki, K. (2007). Gene networks inolved in drought stress response and tolerance. *J. EXP. Bot.* **58**: 221-227.

Shinozaki, K., Yamaguchi-Shinozaki, K. and Seki, M. (2003). Regulatory network of gene expression in the drought and cold stress responses. *Curr Opin. Plant Biol.* **6**: 1-8.

Sieg, F., Schröder, W., Schmitt, J.M. and Hincha, D.K. (1996). Purification and Characterization of a Cryoprotective Protein (Cryoprotectin) from the Leaves of Cold-Acclimated Cabbage. *Plant Physiol.* **111**: 215-221.

Smallwood, M. and Bowles, D.J. (2002). Plants in a cold climate. *Philos Trans R Soc Lond B Biol. Sci.* **357**: 831-847.

Uversky, V.N. and Dunker, A.K. (2010). Understanding protein non-folding. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics **1804**: 1231-1264.

Snowdon, R.J., Friedrich, T., Friedt, W. and Kohler, W. (2002). Identifying the chromosomes of the A- and C-genome diploid *Brassica* species *Brassica rapa* (syn. campestris) and *Brassica oleracea* in their amphidiploid *Brassica napus*. *Theor. Appl. Genet.* **104** (533–538).

Sonnichsen, F.D., Van Eyk, J.E., Hodges, R.S. and Sykes, B.D. (1992). Effect of Trifluoroethanol on Protein Secondary Structure : An NMR and CD Study using a Synthetic Actin Peptide. *Biochemistry* **31**: 8790–8798.

Sreerama, N., Venyaminov, S.Y. and Woody, R.W. (2001). Analysis of protein circular dichroism spectra based on tertiary structure classification. *Anal. Biochem.* **299**: 271–274.

Sreerama, N., Venyaminov, S.Y., and Woody, R.W. (2000). Estimation of protein secondary structure from CD spectra: Inclusion of denatured proteins with native proteins in the analysis. *Anal. Biochem.* **287**: 243–251.

Sreerama, N. and Woody, R.W. (2000a). Circular dichroism of peptides and proteins. *In "*Circular dichroism: Principles and applications, 2nd ed. (eds. N.Berova *et al*.) Wiley, New York: 601–620.

Sreerama, N., Woody, R.W. (2004). On the analysis of membrane protein circular dichroism spectra. *Protein Science* **13**: 100–112.

Sror, H., Tischendorf, G., Sieg, F., Schmitt, J.M. and Hincha, D.K. (2003). Cryoprotectin protects thylakoids during a freeze-thaw cycle by a mechanism involving stable membrane binding. *Cryobiology* **47**: 191-203.

Steponkus, P., Uemura, M. and Webb, M. (1993). A contrast of the cryostability of the Plasma membran of winter rye and spring oat: tow species that widely differ in their freezing tolerance and plasma membrane lipid composition. *In*: Steponkus P.L. (ed): Advances in Low-Temperatur Biology. *JAI Press, London* **2**: 183-210.

Steponkus, P.L. (1984). Role of the plasma membrane in freezing injury and cold acclimation. *Annu. Rev. Plant Physiol.* **35**: 543–584.

Steponkus, P.L. and Lynch, D.V. (1989). Freeze-thaw-induced destabilization of the plasma membrane and the effects of cold acclimation. *J. Bioenerg Biomembr.* **21**: 21-41.

Steponkus, P.L., Lynch, D.V. and Uemura, M. (1990). The influence of cold acclimation on the lipid composition and cryobehaviour of the plasma membrane of isolated rye protoplasts. Philos Trans R Soc Lond B Biol. Sci. **326:** 571-583.

Steponkus, P.L., Uemura, M., Joseph, R.A., Gilmour, S.J. and Thomashow, M.F. (1998). Mode of action of the *cor15a* gene on the freezing tolerance of *Arabidopsis thaliana*. *Proc Natl Acad. Sci. U. S. A* **95**: 14570–14575.

Steponkus, P.L. and Webb, M.S. (1992). Freeze-induced dehydration and membrane destabilization in plants. *In:* G. Somero, B. Osmond, eds, Water and Life: Comparative Analysis of Water Relationships at the Organismic, Cellular and molecular Level. Springer-Verlag, Berlin: 338–362.

Sun, Y. and MacRae, T.H. (2005). Small heat shock proteins: molecular structure and chaperone function. *Cellular and Molecular Life Sciences CMLS* **62**: 2460-2476.

Thalhammer, A., Hundertmark, M., Popova, A.V., Seckler, R. and Hincha, D.K. (2010). Interaction of two intrinsically disordered plant stress proteins (COR15A and COR15B) with lipid membranes in the dry state. *Biochim Biophys. Acta* **1798**: 1812-1820.

Thomashow, M.F. (1998). Role of Cold-Responsive Genes in Plant Freezing Tolerance. *Plant Physiol*. **118**: 1-8.

Thomashow, M.F. (2001). "So what's new in the field of plant cold acclimation? Lots!" .*Plant Physiol.* **125**: 89-93.

Thomashow, M.F. (1994). A*rabidopsis thaliana* as a model for studying mechanisms of plant cold tolerance. *In:*"*Arabidopsis*" A.E. Meyerowitz and C. Somerville (eds.), Cold Spring Harbor Laboratory Press, New York: 807-834.

Thomashow, M.F. (1999). PLANT COLD ACCLIMATION: Freezing Tolerance Genes and Regulatory Mechanisms. *Physiol Plant Mol. Biol.* **50**: 571-599.

Thomashow, M.F. (1990). Molecular genetics of cold acclimation in higher plants. *Advances in Genetics* **28**: 99-131.

Tompa, P. (2002). Intrinsically unstructured proteins. *Trends Biochem.Sci.* **27**: 527–533.

Tompa, P., Bánki, P., Bokor, M., Kamasa P., KovácsLasanda, G. and Tompa, K., (2006). Protein-water and protein-buffer interactions in the aqueous solution of an intrinsically unstructured plant dehydrin: NMR intensity and DSC aspects. *Biophys J.* **91**: 2243–2249.

Tompa, P. and Csermely, P. (2004). The role of structural disorder in the function of RNA and protein chaperones. *FASEB J.* **18**: 1169–1175.

Tompa, P. Szasz, C. and Buday, L. (2005). Structural disorder throws new light on moonlighting. *Trends Biochem.Sci.* **30**: 484–489.

Towbin, H., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Nat. Acad. Science U.S.A* **76**: 4350-4354.

Tunnacliffe, A. and Wise, M. (2007). The continuing conundrum of the LEA proteins. *Naturwissenschaften* **94**: 791-812.

Uemura, M., Gilrnour, S.J., Thomashow, M.F. and Steponkus, P.L. (1996). Effects of COR6.6 and CORl5am polypeptides encoded by *cor* (Cold-Regulated) genes of *Arabidopsis thaliana* on the freeze-induced fusion and leakage of liposomes. *Plant Physiol.* **111**: 313-327.

Uemura, M. and Steponkus, P.L. (1994). A Contrast of the Plasma Membrane Lipid Composition of Oat and Rye Leaves in Relation to Freezing Tolerance. *Plant Physiol.* **104**: 479-496.

Uemura, M. and Yoshida, S. (1984). Involvement of Plasma Membrane Alterations in Cold Acclimation of Winter Rye Seedlings (Secale cereale L. cv Puma). *Plant Physiol.* **75**: 818-826.

Uversky, V.N. and Dunker, A.K. (2010). Understanding protein non-folding. Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics **1804**: 1231-1264.

Van Buskirk, H.A. and Thomashow, M.F. (2006). *Arabidopsis* transcription factors regulating cold acclimation. *Physiol. Plant Mol. Biol.* **126**: 72-80.

Vinocur, B. and Altman, A. (2005). Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current Opinion in Biotechnology* **16**: 123-132.

Vogel, J.T., Zarka, D.G., Van Buskirk, H.A., Fowler, S.G. and Thomashow, M.F. (2005). Roles of the CBF2 and ZAT12 transcription factors in configuring the low temperature transcriptome of *Arabidopsis*. *Plant J.* **41**: 195–211.

Walsh, J.B. (1995). How often do duplicated genes evolve new functions? *Genetics* **139**: 421–428.

Webb, M.S., Gilmour, S.J., Thomashow, M.J. and Steponkus, P.L. (1996). Effects of COR6.6 and COR15am polypeptides encoded by *cor* (Cold-Regulated) genes of *Arabidopsis thaliana* on dehydration-induced phase transitions of phospholipid membranes. *Plant Physiol. Biochem.* **111**: 301-312.

Webb, M.S. and Green, B.R. (1991). Biochemical and biophysical properties of thylakoid acyllipids. *Biochim Biophys Acta* **1060**: 133-158.

Webb, M.S. and Steponkus, P.L. (1993). Freeze-induced membrane ultrastuctural alterations in rye (*Secale cereale*) leaves. *Plant Physiol.* **101**: 171-177.

Webb, M.S., Uemura, M. and Steponkus, P.L. (1994). A comparison of freezing injury in oat and rye: Two cereals at the extremes of freezing tolerance. *Plant Physiol.* **104**: 467-478.

Weiser, C.J. (1970). Cold resistance and injury in woody plants. *Science* **169**: 1269-1278.

Weretilnyk, E., Orr, W., White, T.C., Iu, B. and Singh, J. (1993). Charaterization of three related Iow-temperature-regulated cDNAs from winter *Brassica napus*. *Plant Physiol.* **101**: 171-177.

Werner, A.K., Romeis, T. and Witte, C.-P. (2010). Ureide catabolism in *Arabidopsis thaliana* and *Escherichia coli*. *Nat. Chem. Biol.* **6**: 19-21.

White, T.C., Simmonds, D., Donaldson, P. and Singh, J. (1994)**.** Regulation of BN115, a low temperature responsive gene from winter *Brassica napus*. *Plant. Physiol.* **106**: 917-928.

Williams, P.H. (1989). Wisconsin Fast Plants Manual. Carolina Biological Supply Company, Burlington, North Carolina, *U.S.A.*

Wisniewsk, M., Webb, R., Balsamo, R., Close, T.J., Yu X.M. and Griffith, M. (1999). Purification, imunolocalization, cryoprotective, and antifreeze activity of PCA60: a dehydrin from peach (*Prunus persica*). *Physiol. Plant Mol. Biol.* **105**: 600–608.

Xin, Z. and Browse, J. (2000). Cold comfort farm: the acclimation of plants to freezing temperatures. *Plant Cell Environ.* **23**: 893-902.

Xiong, L., Schumaker, K.S. and Zhu, J.K. (2002b). Cell signaling during cold, drought, and salt stress. *Plant Cell (Suppl)* S165-S183.

Yang, J.T., Wu, C.-S.C. and Martinez, H.M. (1986). Calculation of protein conformation from circular dichroism. *Methods Enzymol.* **130**: 208–269.

Yamaguchi-Shinozaki, K. and Shinozaki, K. (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. *Annu. Rev. Plant Biol.* **57**: 781 - 803.

Zhang, Z., Schwartz, S., Wagner, L. and Miller, W. (2000). A greedy algorithm for aligning DNA sequences. *J Comput. Biol.* **7**: 203 - 214.