



Dehydrins (LTI29, LTI30, and COR47) from *Arabidopsis thaliana* expressed in *Escherichia coli* protect thylakoid membranes during freezing

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Abstract: As the name dehydrins implies, these proteins are typically expressed in response to dehydration, which can be caused by drought, osmotic stress, or freezing temperatures.¹ In general, dehydrins occur in plants as multi-gene families. Four *Arabidopsis* dehydrins (LTI29, ERD14, COR47 and RAB18) were tested for protection of thylakoid membranes during freeze–thaw cycles *in vitro*.^{2,3} The first reported results showed that dehydrins LTI29, ERD14, COR47 have cryoprotective activity while RAB18 did not protect the thylakoid membranes at low temperatures. The cryoprotective activity reached a maximum of 50–60 % at a protein concentration of 140–250 $\mu\text{g mL}^{-1}$ in the assay. A contribution of dehydrins to freezing tolerance *in vivo* is supported by the observation of Nylander *et al.*⁴ that LTI29 and COR47 are cold induced at the mRNA and protein expression level.

Keywords: *Arabidopsis thaliana*; dehydrins; freezing tolerance; thylakoid; cold acclimation.

INTRODUCTION

Dehydration is a common process during seed maturation, which is realized by programmed expression of specific genes.⁵ Nowadays, genes encoding dehydrins have been cloned from numerous plant species belonging to diverse groups such as angiosperms, gymnosperms, mosses and lycopods.⁶ Dehydrins are widely distributed in plants. They significantly vary in amino acid composition (from 100 to 600 amino acid residues) and in molecular mass. The amino acid composition of these proteins is characterized by a high content of charged and polar residues, which determine their biochemical properties, including thermostat-

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bility.^{7,8} The features of dehydrin proteins have recently been summarized in a review by Kosova *et al.*⁹

Structure of Arabidopsis thaliana dehydrins (RAB18, LTI29, LTI30, and COR47)

Isolation and purification of native arabidopsis dehydrins¹⁰ allowed an investigation of their biochemical properties *in vitro*. Generally, arabidopsis dehydrins are enriched in glycine and lysine residues, but lack cysteine and tryptophan.¹¹ For example, in arabidopsis LTI30 dehydrin with a molecular mass of 21 kD, glycine, histidine, lysine, and threonine represent 56 % of the total amino acids, whereas cysteine, tryptophan, arginine, and valine are not found. This explains the highly hydrophilic nature of this protein. Dehydrins are not very likely to form oligomers and they are intrinsically unstructured proteins.

Cryoprotective activity of dehydrins

Many studies reported a positive correlation between the accumulation of dehydrin transcripts or proteins and the tolerance to freezing, drought, and salinity.¹² Puhakainen *et al.*¹ provided data that overexpression of multiple Arabidopsis dehydrin genes, such as LTI29 and LTI30, resulted in an increased freezing tolerance and improved survival under exposure to low temperatures, demonstrating that dehydrins do contribute to freezing tolerance.

One of the proteins with proved cryoprotective activity is a plant lipid-transfer protein called cryoprotectin (CPP).¹³ This protein was isolated from cold acclimated spinach and cabbage leaves that prevented inactivation of cyclic photophosphorylation in spinach thylakoid membranes during freeze–thaw cycles to –25 °C.¹⁴ 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.¹⁵ In the present study, cryoprotectin from cabbage (*Brassica oleracea*) was used as a positive control regarding cryoprotective activity.¹⁶ The aim of the study was to test the cryoprotective activity of *Arabidopsis thaliana* dehydrins LTI29, ERD14, COR47 and RAB 18 on isolated spinach thylakoid membranes during freeze–thaw cycles.

MATERIALS AND METHODS

Spinach (*Spinacia oleracea* L. cv Monnopa) was grown under non-hardening conditions in a growth chamber at 25/15°C (day/night) temperature and 50 % of relative humidity. Cabbage (*Brassica oleracea* L. cv Grüfiwi) was grown in the garden for several months and then transferred to pots. The plants were harvested and the leaves used directly for protein extraction.

Bacterial strain: Escherichia coli M15[pREP4], SG13009[pREP4] Qiagen

For regulated high-level expression with pQE vectors, the cells contained pREP4 plasmid encoding lac repressor in trans, ensuring strictly regulated expression.

Expression vector

The analyzed proteins were expressed using the expression vector pJTS1. This vector was a pQE-60 vector modified by Jan Svensson (Uppsala University, Sweden).¹⁰ Expression vector pQE-60 containing genes for analyzed proteins were transferred to *E. coli* M15 [pREP4], SG13009[pREP4] strain.

Colony screening by PCR

PCR (Table I) was used to screen colonies with the genes of the analyzed proteins. A list of the primers used in this study is shown in the Table II.

TABLE I. PCR conditions

Number of cycle	Temperature, °C	Duration, s
1	94	60
	94	30.
35	– ^a	30
	72	60.

^aDifferent annealing temperatures were used for the amplification of the dehydrins genes: LTI29, 56 °C; ERD14, 50 °C; COR47, 63 °C; RAB18, 63 °C

TABLE II. List of primers used for amplification of dehydrins genes

Primer	Dehydrins gene
PQE F1	5'CCCGAAAAGTGCCACCTG3'
PQE F2	5'CGGATAACAATTTACACAG3'
PQE R	3'GGTCATTACTGGAGTCTTG5'
LTI29	5'GAAAAGAATGGCAGAAGAGTACAAGAACC3'
LTI29	3'TTAATCAGACACTTTTTCTTTCTTCT5'
ERD14	5'CCGCTCGAGAAAAGAATGGCTGAGGAAATCAAGAATG3'
ERD14	3'GCTCTAGATTATTCTTTATCTTTCTTCTCC5'
COR47	5'GAAAAGAATGGCTGAGGAGTACAAGAACAACG3'
COR47	3'TTAATCATCAGACTCTTTTTCTTTCTTCACTTCC5'
RAB18	5'CCGCTCGAGAAAAGAATGGCTCTTACCAGAACCGTCCGTCCAGG3'
RAB18	3'GCTCTAGATTAACGGCCACCACCGGAAGCTTTTCC5'

Protein extraction from E. coli

Dehydrin genes were cloned in a bacterial vector that allowed IPTG-inducible expression of His6-tagged fusion proteins. Bacterial cells were grown in LB medium until an OD_{600} of 0.6 was reached. The cultures were cooled to the induction temperature in ice water. Harvesting of the cells was realized by centrifugation (20 min at 6000 g). The cell pellets were stored at –20 °C.

Cells were lysed by lysozyme treatment followed by sonication.¹⁶ The supernatant and solubilized cell debris were analyzed for the presence of inclusion bodies by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

The proteins were separated on a 16.5 % polyacrylamide resolving gel following the Laemmli procedure.¹⁷ The resolving gel was 7 cm×8 cm×0.1 cm in size and was run at 200 V (constant voltage) for 60–70 min at room temperature. A 10X running buffer according to Laemmli was used. The gel was stained with Coomassie Brilliant Blue R250. Standard

Mark12™ with 12 polypeptides resolved into bands in the range from 2.5 to 200 kDa was used as the standard marker.

Protein purification

The recombinant proteins were efficiently purified with a two-step purification protocol: heat fractionation and immobilized metal ion affinity chromatography (IMAC).¹⁸ Protein concentration was measured using Bradford method.¹⁹ Bovine serum albumin (BSA) was used as the standard. A stock standard solution of 10 mg mL⁻¹ was used to construct a calibration curve by measuring OD_{595} for different BSA concentrations.

Cryoprotectin extraction

Cryoprotectin extraction was performed according to the protocol modified by Hincha and Schmitt.¹⁹

Thylakoid isolation

Thylakoids are sensitive to elevated temperatures, therefore all solutions and glassware should be cooled to 4 °C before use. Isolated membranes should always be kept on ice, preferably in dim light. All centrifugation steps should take place at 4 °C.

Spinach leaves (50 g) were homogenized with 100 mL of homogenization buffer in a blender for approx 10 s. Immediately after, 125 µL of 1 M Na-ascorbate and 340 µL of 1 M cysteine were added to the buffer as these substances are unstable in solution. The homogenate was filtered through a nylon mesh to remove coarse particles. After filtration, the filtrate was centrifuged for 5 min at 2000 g. The pellets were resuspended in approximately 50 mL of washing solution. This was most easily performed using a Pasteur pipette. Centrifugation at 7000g for 5 min was performed with the aim of washing the thylakoid pellet; this procedure was repeated twice. The pellets from the last centrifugation step were resuspended in a minimum volume of washing solution. 10 µL of the thylakoid suspension was mixed with 990 µL of 80 % (v/v) acetone and centrifuged for 2 min in a bench-top centrifuge. The absorbance of the supernatant at 663 and 645 nm with 80 % (v/v) was measured using acetone as the reference. Chlorophyll content is calculated as follows:

$$0.1(8.02A_{663} + 20.2A_{645}) = \text{mg chlorophyll mL}^{-1} \quad (1)$$

Freeze-thaw cycle

Thylakoid suspension was diluted with washing buffer to a concentration of at least 1 mg chlorophyll mL⁻¹. The thylakoid suspension (0.5 mL) was mixed with an equal volume of proteins suspension in Eppendorf tubes. The suspension was placed in a freezer at -20 °C for 2 h.

Samples are most conveniently thawed in a water bath at room temperature and should be transferred to an ice bath immediately when the ice in the tubes has melted.

Thylakoid volume measurements

Hematocrit capillaries (Fig. 1) were loaded with diluted thylakoid suspensions after thawing and the capillaries were sealed at one end. Capillaries were centrifuged for 15 min in a hematocrit centrifuge and pellet heights were measured (Fig. 1) with a magnifying glass on a 0.1 mm scale.

The cryoprotective activity (in %) was calculated using the equation:

$$TKV(\text{PP}-20\text{ }^{\circ}\text{C}) - TKV(-20\text{ }^{\circ}\text{C}) / TKV(0\text{ }^{\circ}\text{C}) - TKV(-20\text{ }^{\circ}\text{C}) = X/100 \quad (2)$$

where $TKV(\text{PP}-20\text{ }^{\circ}\text{C})$ is the thylakoid volume in presence of analyzed protein at -20 °C, $TKV(-20\text{ }^{\circ}\text{C})$ is the thylakoid volume without protein at -20 °C and $TKV(0\text{ }^{\circ}\text{C})$ is the thylakoid volume without protein at 0 °C.

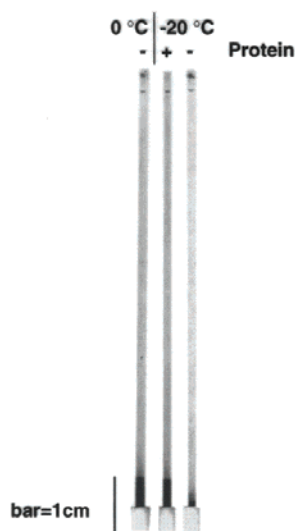


Fig 1. Volumetric test. Hematocrit capillaries were used to measure the cryoprotective activity. The controls at 0 and -20°C show the total volume of the thylakoids at these temperatures after centrifugation; + – the thylakoid volume at -20°C in the presence of dehydrins; - – the thylakoid volume at -20°C without any dehydrin.

Immunoblot analysis

The antibodies for LTI29 and RAB18 dehydrins were developed by J. Svensson (Uppsala University). It was possible to obtain a LTI29 antibody that was specific to LTI29 and the previously made RAB18 antibodies were only recognized by RAB18.²⁰ Both antibodies were directed against the conservative K-segment of the dehydrins. The analyzed protein (10 μg) was subjected to 16.5 % SDS-PAGE and transferred onto nitrocellulose membranes (Amersham Biosciences Inc.). The nitrocellulose membranes were incubated with primary antibody (diluted 1:250 to 1:1000 in blocking buffer) for 2 h in blocking buffer at room temperature, and 1:2000 dilution for affinity-purified rabbit polyclonal to green fluorescent protein (GFP) antibody (Abcam). Blots were then probed with peroxidase-labeled anti-rabbit secondary antibodies (Dako) at 1:2000 dilution. The signals were visualized using an enhanced chemiluminescence reagent (ECL, Amersham Biosciences Inc.) and exposed onto an ECL Hyperfilm (Amersham Biosciences Inc.).

RESULTS AND DISCUSSION

Transformation and checking by colony PCR

Escherichia coli M15 strain was transformed and screened for positive clones by PCR constructed primers for each gene individually. Previously, a PCR program was designed (suitable annealing temperatures) for all the analyzed sequences (Table II).

The PCR products were analyzed by 2 % agarose gel electrophoresis (Fig. 2). For each analyzed gene, the corresponding PCR product in base pairs length was confirmed.

Dehydrins expression

The highest production of soluble dehydrins was obtained by inducing expression of the cloned dehydrin genes with IPTG (isopropylthio- β -galactoside)

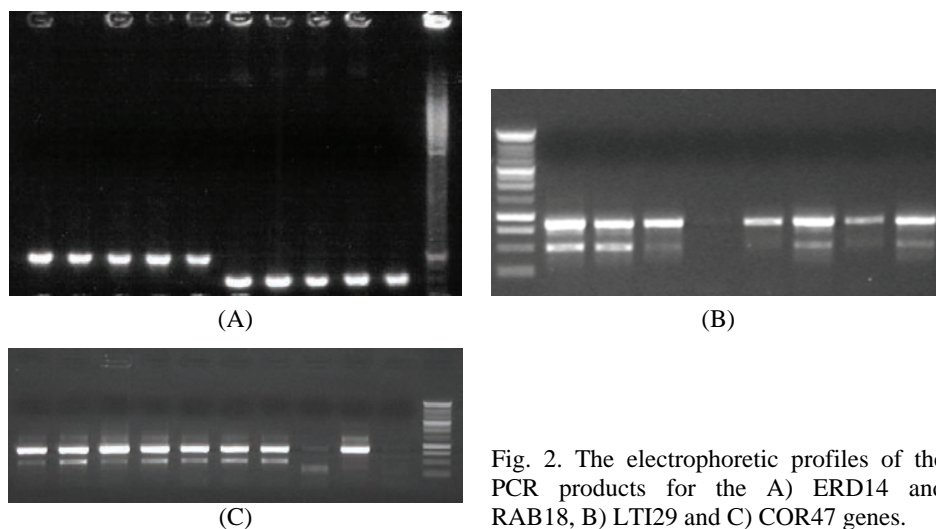


Fig. 2. The electrophoretic profiles of the PCR products for the A) ERD14 and RAB18, B) LTI29 and C) COR47 genes.

when the cells reached an OD_{600} of 0.5–0.7. Overproduction of recombinant proteins in *E. coli* often results in the formation of insoluble inclusion bodies. After resuspension in 20 mM NaH_2PO_4 , pH 6.0, it was clear that the recombinant proteins were localized in the supernatant and did not form inclusion bodies. For heat fractionation, the lysate was placed in a water bath at 90 °C for 8 min. Approximately 80 % of the contaminating proteins precipitated during the heat fractionation, while the dehydrins remained heat stable. Using concentration filters (Promega), the final concentration of the analyzed proteins was doubled (Figs. 3 and 4). Heat treatment was followed by immobilized metal ion affinity chromatography (IMAC).¹⁸ Purification according to this procedure showed additional bands (Figs. 3 and 4) originating from crude bacterial proteins which had no cryoprotective activity (negative control). The yields of purified proteins were between 4 and 15.5 mg L^{-1} of bacterial culture, which was sufficient for further biochemical studies. The estimated molecular mass values for the analyzed proteins according to SDS–PAGE were the following: LTI29, 47 kDa; ERD14, 34 kDa; COR47, 54 kDa; RAB18, 25 kDa.

Protein desalting

The analyzed proteins were desalted using PD10 desalting columns (Promega). Protein concentration and conductivity from small aliquots (50 μL) were measured simultaneously. It was shown that proteins LTI 29 and COR 47 (Fig. 5) flowed through the column after 1.5 mL eluting buffer and the highest concentration of the proteins was reached at 2.5–3.5 mL. Elution kinetics for all proteins were different because of their different size (Fig. 5).

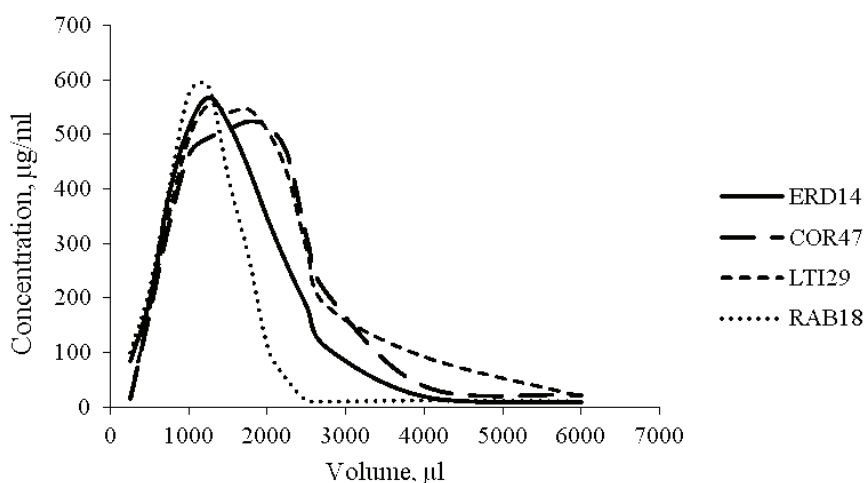
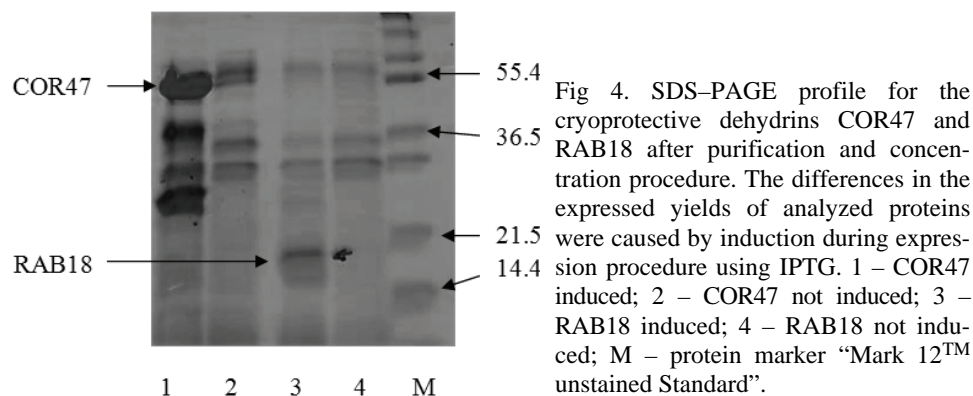
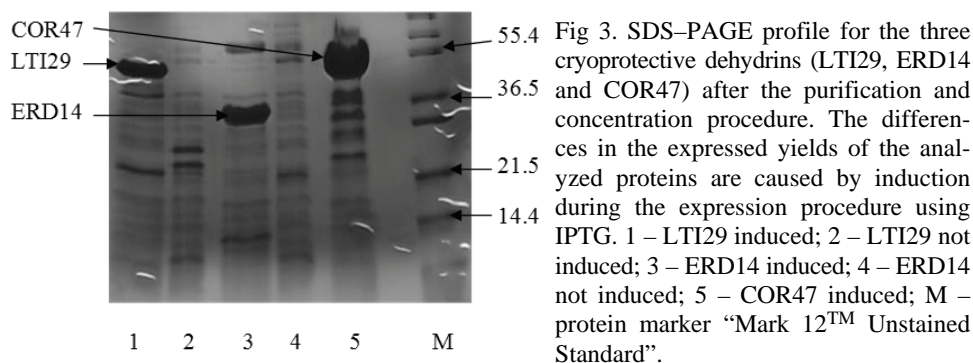


Fig 5. Protein desalting using desalting PD 10 columns. Using desalting columns, ammonium sulfate was removed from the solutions containing the analyzed proteins. It was shown that proteins LTI 29, COR 47 and ERD 14 had different elution kinetics to that of RAB18 because of the different dimensions of the analyzed proteins.

Cryoprotective activity

During the period of plant acclimatization to low temperatures, it was shown¹² that the dehydrins were expressed, which indicates a possible cryoprotective activity of these proteins. A freezing test, which was based on measuring the ability of proteins to protect thylakoids during a freeze–thaw cycle, was used in this study.¹⁹ As a result, cryoprotective activity was evidenced in three out of four analyzed dehydrins. (Table III).

In addition to these results, dehydrin RAB18 had a small percentage of cryoprotective activity over the level of the negative control. As a negative control, a non-induced crude bacterial extract boiled, purified and concentrated using the previously described procedure, which showed no cryoprotective activity, was used. As a positive control, a CPP (cryoprotectin) was used.¹³ In this study, it was found that the analyzed dehydrins (LTI29, ERD14, and COR47) from *Arabidopsis thaliana* have cryoprotective activity in various percentages (Fig. 6). Cryoprotective activity was individually measured 10 times per each dehydrin.

TABLE III. Cryoprotective activity of the analyzed dehydrins and CPP protein in %; SD – standard deviation; AV – average value; Cont. 0 °C and Cont –20 °C – measured cryoprotective activity at 0 and –20 °C without added proteins in the suspension

Dehydrin/protein	1	2	3	4	SD	AV
ERD 14	47.4	43.6	56.8	53.1	5.89	49.3
RAB 18	7.6	15.2	3.8	5.7	4.99	8.1
LTI 29	47.4	36	43.6	45.5	4.9	43.1
COR 47	41.7	45.5	43.6	30.3	6.8	40.2
Cont. 0 °C	115.6	94.8	96.7	92.9	10.5	100
Cont. –20 °C	0.0	1.9	0.0	1.9	1.55	0.95
CPP	59.4	55	62	60.8	4.94	59.3

The protein concentration is a relevant factor for the level of cryoprotective activity (Fig. 7). With increasing initial protein concentration, the cryoprotective activity increased. Saturation was reached at an activity of 50–60 %.

The possible mechanism of dehydrin cryoprotection is still unknown. Some authors reported that dehydrins have a binding ability to lipid vesicles,²⁰ therefore, the binding ability of dehydrins LTI29 and RAB18 to thylakoid membranes was analyzed. Thylakoids from non-hardened spinach leaves were incubated with dehydrins LTI29 and RAB18 and then subjected to three rounds of centrifugation and resuspension in a protein-free solution.

Immunoblot analyses of supernatant after several washing steps are shown in Fig. 8. After several washing steps, it was shown that the supernatant was free of dehydrins, while dehydrin with binding ability to thylakoid membrane was assumed to be found.

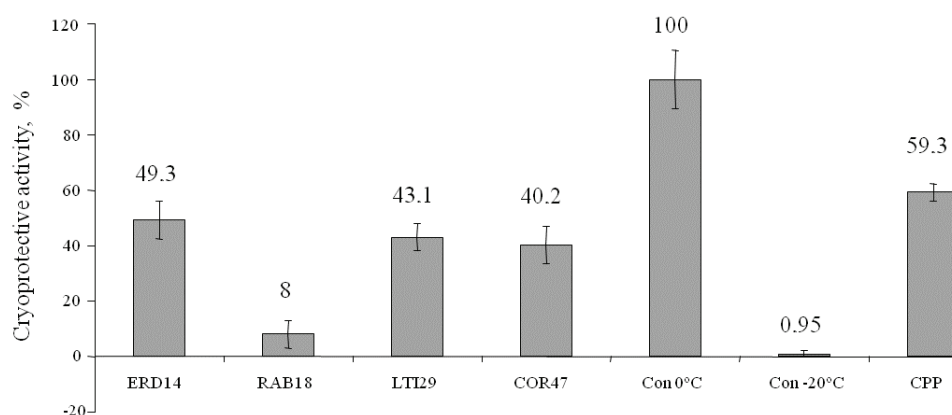


Fig 6. Cryoprotective activity of four analyzed dehydrins. Using the “freezing test”, the results showed that the four dehydrins had different cryoprotective activities. It was also revealed that dehydrin RAB18 had a smaller percentage of cryoprotective activity. A crude extract of cold hardened *Brassica oleracea* containing cryoprotectin¹⁶ was used as the positive control (CPP). As a negative control, a non-induced crude bacterial extract boiled, purified and concentrated using the standard procedure, which showed no cryoprotective activity, was used (Con -20 °C).

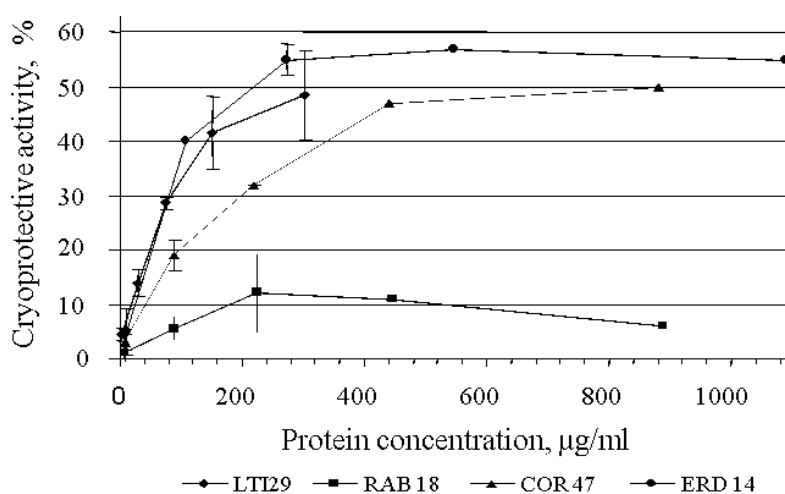


Fig 7. The cryoprotective activity as a function of protein concentration. The dependences of cryoprotective activities on the concentration of the protein are linear only for low protein concentrations; at higher protein concentrations, there are clear saturation points for the cryoprotective activities of the proteins.

Immunoblot analyses showed (Fig. 8, line 4) that the protein LTI29 was still detected after several washing steps. In the parallel experiment with protein RAB18 (no cryoprotective activity), the protein was not visible. The result of this experiment clearly shows the presence of binding ability of LTI29 protein to the

thylakoid membranes, which could be linked to the cryoprotective activity of the protein. Immunoblot analysis of thylakoid membranes showed that there was no protein present that could react with the antibody.

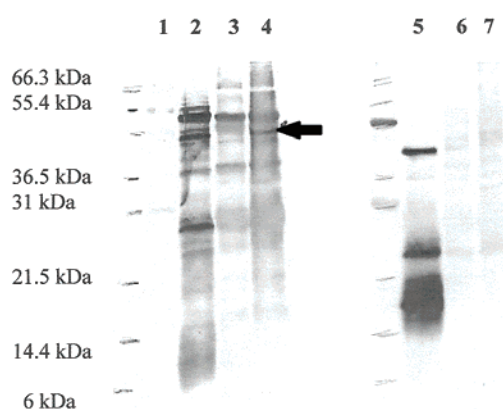


Fig 8. Binding ability of dehydrins LTI29 and RAB18. Immunoblot analyses indicated to binding abilities of dehydrins to the thylakoid membrane, which were in correlation with their cryoprotective activity. 1. PM – protein marker; 2. LTI29 – expressed protein LTI29 from *E. coli*; 3. Thyl. thylakoid proteins; 4. Thyl.+ LTI29 (after washing); 5. RAB18 – expressed protein RAB18 from *E. coli*; 6. Thyl. thylakoid proteins; 7. Thyl.+RAB18 (after washing). The arrow shows the position of dehydrin LTI29 after several washing steps.

CONCLUSIONS

The results show, for the first time, that three out of four analyzed proteins from *Arabidopsis thaliana* expressed in *Escherichia coli* protect thylakoid membranes during a freeze–thaw cycle *in vitro* (cryoprotective activity), which indicates the possible function of these proteins under stress conditions. The highest cryoprotective activity was shown by the ERD14 dehydrin (49 %), while dehydrins LTI29 and COR47 showed similar levels of cryoprotective activity (43 and 40 %, respectively). In this study, no cryoprotective activity for the protein RAB18 was evidenced. It was also demonstrated that most of the proteins from the bacterial supernatant become denatured due to the heat treatment process, while dehydrins remained stable. This was taken to be an important step in the purification of the dehydrins. In addition, the yield of the proteins was satisfactory although differences in the yields among the analyzed proteins were found. Immunoblot analyses indicated that the binding ability of dehydrins to the thylakoid membrane was in correlation with their cryoprotective activity. Dehydrin RAB18, which showed no cryoprotective activity, did not bind to the thylakoid membranes, according to the present results. This suggests that the binding of analyzed proteins to the cell membrane is an important prerequisite for cryoprotective activity. The protein concentration is a relevant factor for the level of cryoprotective activity since cryoprotective activity increases with increasing initial protein concentration. Further studies should be performed with the aim of investigating the possible mechanism of cryoprotective activity of the dehydrins used in this study.

ИЗВОД

ДЕХИДРИНИ (LTI29, LTI30, COR47) ИЗ *Arabidopsis thaliana* ЕКСПРИМИРАНИ У *Escherichia coli* ШТИТЕ ТИЛАКОИДНЕ МЕМБРАНЕ ТОКОМ СМРЗАВАЊАVLADAN BOZOVIC¹, JAN SVENSSON², JURGEN SCHMITT³ И CARSTEN KOHN³¹University of Montenegro, Biotechnical Faculty, Podgorica, Montenegro, ²Uppsala Genetic Center, Swedish University of Agricultural Sciences, Uppsala, Sweden и ³Institute for Plant Physiology and Microbiology – Department of Biology, Chemistry and Pharmacy – FU Berlin, Berlin, Germany

Као што им само име говори, дехидрини су протеини који се експримирају као одговор на дехидратацију изазвану сушом, осмотским стресом или замрзавањем. Дехидрини су у биљкама кодирани мултигенским фамилијама. Заштитна улога четири дехидрина биљке *Arabidopsis* (LTI29, ERD14, COR47 и RAB18) приликом замрзавања тилакоидних мембрана је испитана *in vitro*. Наши резултати су показали да дехидрини LTI29, ERD14 и COR47 имају криопротективно дејство, док тај ефекат нема RAB18. Максимална криопротективна активност (50 до 60 %) је постигнута при концентрацији протеина од 140–250 µg mL⁻¹ у тесту. О утицају дехидрина говоре и литературни подаци, према којима је експресија LTI29 и COR47, на РНК и протеинском нивоу, подстакнута ниским температурама.

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