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DEHYDRINS FROM ARABIDOPSIS THALIANA EXPRESSED IN E. COLI PROTECT MEMBRANES DURING FREEZING

Vladan BOZOVIC[1]*, Jan SVENSSON[2], Jurgen SCHMITT[3], Carsten KOHN[3]

[1]University of Montenegro, Biotechnical Faculty, Podgorica, Montenegro
[2]Uppsala Genetic Center, Swedish University of Agricultural Sciences, Uppsala, Sweden
[3]Institute for Plant Physiology and Microbiology – Department of Biology, Chemistry and Pharmacy - FU Berlin, Germany
*(Corresponding author e-mail: vladan.bozovic@yahoo.com)

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. In general, dehydrins occur in plants as multi-gene families. *Arabidopsis* dehydrins (LTI29, ERD14, COR47 and RAB18) have been tested for protection of thylakoid membranes during freeze thaw cycle *in vitro*. The results obtained in this study show that dehydrins LTI29, ERD14, COR47 protect thylakoid membranes at low temperatures. A correlation between level of cryoprotective activity and protein concentration has been highlighted. The preliminary results of this study indicate a possible mechanism of cryoprotection in plants.
Keywords: Arabidopsis thaliana; dehydrins; freezing tolerance; thylakoid; cold acclimation.

Introduction

Acclimation capacity of plants

Plants differ in their capacity to cope with sub-optimal temperatures. Chilling sensitive plants, often growing in tropical areas, are injured at temperatures just below +10 °C. Chilling tolerant plants can tolerate low, non-freezing temperatures, but are killed in temperatures a few degrees below zero. Plants that can tolerate freezing temperatures employ two major strategies. They either avoid freezing or tolerate extracellular freezing (Sakai and Larcher, 1987).

Dehydrins (main characteristics)

Dehydration is a common process during seed maturation, which is realized by programmed expression of specific genes. The phytohormone abscisic acid (ABA) plays a key role in the regulation of this process. Several genes specifically expressed in this ontogenetic period have been recognized. They include genes encoding LEA (Late Embryogenesis Abundant) proteins. LEA proteins have been found in ABA treated vegetating plants and also under the stress conditions that result in cellular dehydration induced by drought, salinity, or low temperatures. One group of such genes encodes dehydrins (DHN), known also as group 2 late embryogenesis abundant (LEA) proteins. Nowadays, genes encoding DHNs have been cloned from numerous plant species belonging to such diverse groups as angiosperms, gymnosperms, mosses and lycopods (Svensson et al., 2002).

Structure of *Arabidopsis thaliana* dehydrins

(RAB18, LTI29, LTI30, and COR47). Isolation and purification of native Arabidopsis dehydrins (Svensson et al., 2000) allowed the investigation of their biochemical properties *in vitro*. Generally, Arabidopsis dehydrins are enriched with glycine and lysine residues, but they lack cysteine and tryptophan (Wisniewski et al., 1999).

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 (Rodriguez et al., 2005; Nylander et al., 2001; Houde et al., 1992.

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

Materials and methods

Plant sources utilized in this study

Spinach *(Spinacia oleracea* L. cv Monnopa) was grown under non-hardening conditions in a growth chamber with 12 h of light at 150 pmol quanta m-’sP1 at 25 °C and 12 h of dark at 15°C at 50% RH. (Production: Julius Wagner GmbH)

Cabbage *(Brassica oleracea* L. cv Grüfiwi) was grown in the garden for several months and then transferred to pots. Plants were harvested, and leaves were either used directly for protein extraction or were stored frozen at -20°C.

Bacterial strain

E. coli M15[pREP4], SG13009[pREP4] Qiagen was used for regulated high-level expression with pQE Vectors (Cells contain pREP4 plasmid encoding lac repressor in trans, ensuring tightly regulated expression).

Expression vector

Analyzed proteins have been expressed using the vector pJTS1. This vector was modified pQE-60 vector by Jan Svensson (Sweden, Upsala University) (Svensson, 2000).

Colony screening by PCR

PCR is used to amplify specific regions of a DNA strand. The set of primers used in this study are listed in Tab.1.

Tab. 1. Lists of oligonucleotides (primers) used in this study

PQE F1 5’CCCGAAAAGTGCCACCTG3’
PQE F2 5’CGGATAACAATTTCACACAG3’
PQE R 3’GGTCATTACTGGAGTCTTG5’
LTI29 5'GAAAAGAATGGCAGAAGAGTACAAGAACACC3'
LTI29 3'TTAATCAGACACTTTTTCTTTCTTCTCT5'
ERD14 5´CCGCTCGAGAAAAGAATGGCTGAGGAAATCAAGAATG3´

ERD14 3´GCTCTAGATTATTCTTTATCTTTCTTCTCC5´
COR47 5'GAAAAGAATGGCTGAGGAGTACAAGAACAACG3'
COR47 3'TTAATCATCAGACTCTTTTTCTTTCTTCACTTCC5'
RAB185'CCGCTCGAGAAAAGAATGGCGTCTTACCAGAACCGTCCGTCCAGG3'
RAB183'GCTCTAGATTAACGGCCACCACCGGGAAGCTTTTCC5'

Protein expression

The optimal OD value depends on the method and the medium. For flask cultures using LB-medium an **OD_{600}** of **0.6** is recommended.

Protein extraction from *E. coli*

Cells were lysed by a *Lysozyme treatment plus sonication* as follows: add lysozyme to a final concentration of 100 µg/ml from a freshly prepared 10 mg/ml stock in water. Incubate at 30°C for 15 min. Mix by swirling and sonicate on ice using a microtip with the power level set between 4–5. Sonicate 4 times for 45 sec. Incubate at 90°C for 8 min. Take a 1.5 ml sample of the lysate and centrifuge at 14,000 g for 10 min to separate the soluble and insoluble fractions. Transfer 100 µl of the soluble supernatant to a new tube. Add 100 µl of 2X Sample Buffer (2X SB = 100 mM DTT, 2% SDS, 80 mM Tris-HCl, pH 6.8, 0.006% bromophenol blue, 15% glycerol) to 100 µl soluble fraction sample. Store at –20°C until SDS-PAGE analysis. Supernatant and solubilised cell debris were analyzed for the presence of inclusion bodies.

Cryoprotective leaf proteins extraction and Freezing test

The extraction of leaf proteins and the freezing test were carried out by following the procedures described by Hincha and Schmitt (1992)

Freezing test

All centrifugation steps take place at 4 °C.

1. *Thylakoid isolation:* 50 g of spinach leaves was homogenized with 100 ml of homogenization buffer in a blender for approx 10 s. 125 µl of 1 M Na-ascorbate and 340 µl of 1M cysteine was added to the buffer immediately before use, as these substances are unstable in solution. Centrifugation at 7000 g for 5 min was done with the aim of washing thylakoid pellet; this procedure was repeated twice. 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 benchtop centrifuge. Absorbance of the supernatant at 663 and 645 nm with 80 % (v/v) acetone as the reference was measured. Chlorophyll content is calculated as follows:
   Chlorophyll content

2. (8.02 * A663 + 20.2 * A645) * 0.1 = mg Chlorophyll / ml. Hincha and Schmitt (1992)

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

3. 0.5 ml of the thylakoid suspension was mixed with an equal volume of proteins suspension in Eppendorf tubes.
4. The suspension was placed in a freezer at -20 °C for 2 h.
5. 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

Aliquots of thylakoids in cryopreservation solution were mixed with sucrose solutions ranging in concentration from 20 to 500 mM sucrose finally. All sucrose solutions were made in 5 mM $MgCl_2$.

The osmolality of the resulting solutions was measured with an osmometer. These measurements are made easier by using the cryopreservation solution diluted 1:1 with the washing solution instead of thylakoids. The final results will be the same.

Hematocrit capillaries were loaded with the diluted thylakoid suspensions and the capillaries were sealed at one end. Then they were centrifuged for 15 min in a hematocrit centrifuge and pellet heights were measured with a magnifying glass and a 0.1 mm scale.

Fig.1. Volumetric test – Hematocrit capillaries were used to measure cryoprotective activity. Controls at 0 °C and -20 °C show whole volume of thylakoids at these temperatures after centrifugation.

The cryoprotective activity (in percentage) was calculated as follows:

TKV(PP -20°C) - TKV(-20°C) / TKV(0°C) - TKV(-20°C) = X/100

TKV(PP -20°C) - thylakoid volume in presence of analyzed protein at -20°C

TKV(-20°C) - thylakoid volume without protein at -20°C

TKV(0°C) - thylakoid volume without protein at 0°C

Results and discussion

Transformation and checking by colony PCR

E. coli M15 strain was transformed. Screening for positive clones was performed with PCR using constructed primers for each gene individually.
Previously the PCR program was standardized (suitable annealing temperature) for all analyzed genes. 94 °C for 60 sec, 94 °C for 30 sec, * °C for 30 sec 35 cycles, 72 °C for 1 min.
Annealing temperature: (*) LTI29 56 °C; ERD14 50 °C; COR47 63 °C; RAB18 63 °C
PCR products were analysed by agarose gel electrophoresis (Fig. 2).

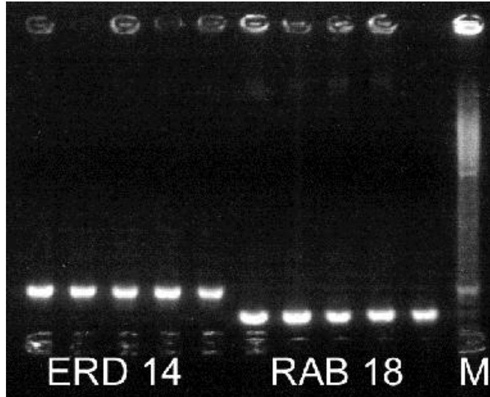

Fig. 2. PCR products for ERD 14 and RAB 18 genes respectively

Dehydrins expression

Maximal production of soluble dehydrins was obtained by inducing expression of the cloned dehydrin genes with IPTG when the cells reached an OD_{600} of 0.5-0.7. Recombinant proteins were localized in the supernatant and did not form inclusion bodies. For heat fractionation, lysates were placed in a 90 °C water bath for 8 min. Approximately 80% of the contaminating proteins precipitated during the heat fractionation. Using concentration filters final concentration was doubled. Cryoprotective dehydrins were analyzed in SDS – PAGE after purification and concentration (Fig. 3).

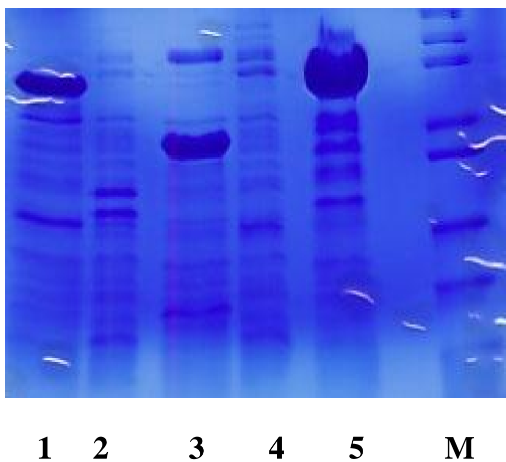

Fig. 3. SDS - PAGE analyses of three cryoprotective dehydrins (LTI29,ERD14 and COR47) after purification and concentration. From gel is visible relation between expression yields of analysed proteins. 1 – LTI29 induced; 2-LTI29 no induced; 3-ERD14 induced; 4-ERD14 no induced; 5-COR47 induced; M-protein marker

Protein desalting

The graph in Fig. 4 was constructed for two analysed proteins LTI 29 and RAB 18.
It was shown that protein LTI 29 flow through column after 1,5 ml eluting buffer and the highest concentration of protein was reached at 2,5 – 3,5 ml. The similar elution kinetic was found for other dehydrins except RAB 18. Elution kinetics for protein RAB 18 is different because of smaller size of this protein.

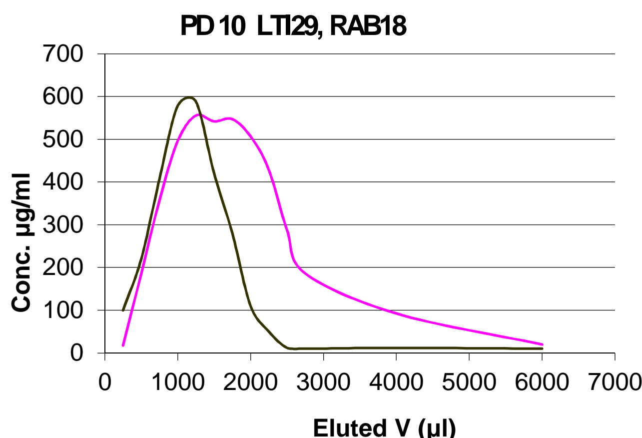

Fig. 4. Protein desalting using desalting columns PD 10. Using desalting columns ammonium sulphate was removed from solution containing analysed proteins. It was shown that proteins LTI 29 and RAB 18 have different elution kinetics because of different dimensions of analysed proteins.

Cryoprotective activity

In previous reports it was shown that dehydrins are expressed during the period of plant acclimatization to low temperatures, which points to possible cryoprotective activity of these proteins (Thomashow, 1990). In this study a freezing test based on measuring the ability of proteins to protect thylakoids during a freeze-thaw cycle was used. The results showed that three of four analyzed dehydrins had cryoprotective activity (Fig. 5). In addition to these results dehydrin RAB18 had a low cryoprotective activity (in %) over the level of the negative control. As negative control non induced crude bacterial extracts were used, which showed no cryoprotective activity. As positive control a CPP was used (Hincha et al., 1996). The test confirmed that analyzed dehydrins (LTI29, ERD14, COR47) from Arabidopsis thaliana have cryoprotective activity.

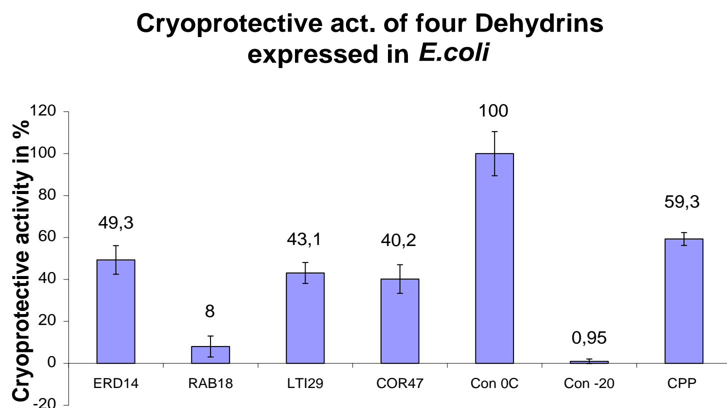

Fig. 5**.** Cryoprotective activity for four analyzed dehydrins expressed in %.

The results of "freezing test" showed that four dehydrins had different cryoprotective activity. It also revealed that dehydrin RAB18 had low cryoprotective activity. A crude extract of cold hardened *Brassica oleracea* containing cryoprotectin (Hincha et al., 1996) was used as a positive control (CPP).
The concentration of proteins is a relevant factor for the level of cryoprotective activity. During increasing of the initial protein concentration, the cryoprotective activity also increased. Saturation was reached at 50% - 60% activity.

Conclusions

The cryoprotective activity was proven for 3 of 4 analyzed recombinant dehydrin proteins (LTI29, COR47 and ERD14) expressed in *E. coli* by using freezing test. For the protein RAB 18 a low cryoprotective activity has been shown in this test. Due to the process of heat treatment most of the proteins from bacterial supernatant become denaturalized, while the dehydrins remain heat stable. This was shown to be an important step in the purification of dehydrins. Also the yield of proteins reached satisfactory level although some differences in the yield among the analyzed proteins were noticed. Cryoprotective activity of dehydrins is in positive correlation to the concentration of the analysed proteins used in the assay.

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