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Detection of dehydrin-like proteins in embryos and endosperm of mature *Euterpe edulis* seeds

V. Panza[1,2], **A. J. Distéfano**[3], **P. Carjuzaa**[2], **V. Láinez**[2], **M. del Vas**[3], and **S. Maldonado**[1,2,*]

[1] Instituto de Recursos Biológicos, Centro de Investigación de Recursos Naturales, Instituto Nacional de Tecnología Agropecuria, Hurlingham, Buenos Aires
[2] Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Buenos Aires
[3] Instituto de Biotecnología, Centro de Investigación de Ciencias Veterinarias y Agronómicas, Instituto Nacional de Tecnología Agropecuria, Hurlingham, Buenos Aires

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Summary. *Euterpe edulis* Martius, a tropical palm species characterized as highly recalcitrant, accumulated dehydrin proteins in both the endosperm and the embryo of the mature seed, as detected by Western blot analysis and immunogold electron microscopy. Three major bands at molecular masses of approximately 16, 18, and 24 kDa were identified in both samples analysed. Immunogold electron microscopy studies detected the presence of dehydrins in the embryo and endosperm. In both cases, dehydrins were immunolocalized in cytoplasm and chromatin. No labelling associated with either membranes or organelles was detected. It is known that dehydrins are produced as part of the developmental program of orthodox seeds and are also present in some recalcitrant seeds of temperate regions. The constitutive presence of dehydrins in embryos of extremely recalcitrant species of tropical origin has not been previously reported.

Keywords: Dehydrin; Western blot; *Euterpe edulis*; Palmito; Recalcitrant seed; Subcellular immunolocalization.

Introduction

The LEA D11 family is an immunologically distinct family of proteins known as dehydrins (Dure 1993), which includes some of the most common dehydration- or low-temperature-induced proteins (Close 1997). The defining feature of dehydrins is the presence of a predicted amphipathic α-helix-forming domain called the K segment (Close 1996). The K segment occurs in one to eleven copies, which are located near the dehydrin carboxyl terminus. The consensus amino acid sequence for angiosperms is TGEKKGIMDKIKEKLPGQH (Close 1996). To date, dehydrins have been identified in over 30 plant taxa with antibodies against this sequence (Close et al. 1993).

Although dehydrins often exhibit tissue and organ specificity, immunolocalizations performed in cotton and maize seeds have shown that they are present in all tissue types (Roberts et al. 1993, Asghar et al. 1994, Danyluk et al. 1994, Close 1996). At the subcellular level, seed dehydrins have been immunolocalized in the cytoplasm of cells next to the shoot and root apex of maize embryo tissues and also in the cytoplasm, cytoskeletal elements, and nucleus of cells belonging to endosperm (aleuronic layer) and scutellar tissues (Asghar et al. 1994, Close 1996).

Euterpe edulis is a tropical species with recalcitrant seeds (Martins et al. 2000, Andrade 2001, Panza et al. 2004). Recalcitrant seeds are those seeds that undergo little or no maturation drying and remain desiccation sensitive both during development and after they are shed (Pammenter and Berjak 1999). According to Kermode (1997), dehydrins are produced as part of the developmental program of orthodox seeds. Dehydrins have also been detected in the recalcitrant seeds of *Acer saccharinum*, *Aesculus hippocastanum*, *Araucaria angustifolia*, *Camellia sinensis*, *Castanea sativa*, and *Poncirus trifoliate*, all of which are of temperate origin (Farrant et al. 1996), but not in those of various tropical wetland recalcitrant species, such as *Avicennia marina*, *Bruguiera cylin-*

* Correspondence and reprints: Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria, C1428EHA, Ciudad Autónoma de Buenos Aires, Argentina.
E-mail: saram@bg.fcen.uba.ar

drica, *B. exaristata*, *B. gymnorrhiza*, *Ceriops tagal*, *Rhizophora apiculata*, *R. mucronata*, *R. stylos*, and *Trichilia dregeana* (Farrant et al. 1996, Han et al. 1997, Kermode 1997). Dehydrins were detected in mature seeds of *Catanospermus australe*, a recalcitrant species tropical in origin, only when individuals had developed in a temperate climate (Farrant et al. 1996, Han et al. 1997). In three other recalcitrant species, dehydrins accumulated to detectable levels only after exposure to additional water loss (*Porteresia coarctata* and *Zizania palustris*) or in response to an increase in abscisic acid accumulation (*Barringtonia racemosa*) (Farrant et al. 1996).

Euterpe edulis occurs in south-eastern Brazil, north-eastern Argentina, and Paraguay. Previous studies of *E. edulis* have reported that the mean moisture content of the whole mature seed is around 48% (wet weight) (Martins et al. 2000, Andrade 2001). In turn, isolated embryos and endosperms have mean moisture contents of 85% and 48% (wet weight), respectively (Panza et al. 2004). In addition, several *E. edulis* embryo tissues have been found to present subcellular features associated with the extremely recalcitrant behaviour known for this seed, such as fresh and highly vacuolated tissues virtually lacking storage reserves with abundant endomembranes and ergastic substances (Panza et al. 2004).

In an effort to understand the seed physiology of *E. edulis* seeds and to identify the causes of its recalcitrant behaviour, we have analysed the presence of dehydrins in the endosperm and embryo. To our knowledge, dehydrins have not been previously detected in any palm seed. The results are compared with published results for recalcitrant species from other families. This study is part of a monographic treatment on the conservation of *E. edulis* seeds.

Material and methods

Plant material

Mature fruits of *Euterpe edulis* Martius were harvested from plants growing at Iguazú National Park, Misiones, Argentina, during August 2001 and 2002 and immediately stored at 4 °C for no longer than 1 week at the Germplasm Bank of the Instituto Nacional de Tecnología Agropecuria, Castelar, Buenos Aires, Argentina. In order to avoid the possible effect of cold temperature on dehydrin synthesis, a new collection was made in August 2005 and 2006, but this time fruits were processed immediately after shedding.

Before use, seeds were manually extracted and the pericarps were removed. Wheat caryopses (*Triticum aestivum* L., cv. Chinese Spring) were provided by Dr. Silvina Lewis (INTA, Castelar) in August 2002.

Protein extraction and Western blot assay

Lyophilized embryo and endosperm tissues (50 mg) were powdered, transferred into Eppendorf tubes (volume, 1.5 ml) and suspended in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride at a ratio of 15 μl/mg of sample. Samples were stirred for 30 min and then centrifuged for 10 min at 10,000 **g**, and the supernatants were collected as soluble fractions. Total soluble protein was heated to 85 °C for 10 min and then centrifuged for 10 min at 10,000 **g**. The heat-stable protein concentration was determined by the Bradford method (Bradford 1976). Proteins were resolved by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli 1970) using a Miniprotean II electrophoresis cell (Bio-Rad, Hercules, Calif., U.S.A.); 6 μg of heat-stable proteins were loaded in each well. Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose membrane (GE Osmonics, Minnetonka, Minn., U.S.A.) by the Bio-Rad Miniprotean II system. The integrity of blotted proteins was confirmed by Ponceau S staining.

After an overnight blocking with 0.5% nonfat dried milk in Tris-buffered saline (TBS) at 4 °C, the membrane was incubated for 2 h with an anti-dehydrin rabbit polyclonal antiserum at a 1 : 1000 dilution in TBS. The antiserum was raised against a consensus peptide sequence usually present at the dehydrin carboxyl terminus (TGEKKGIMD KIKEKLPGQH) (Close et al. 1993) and was kindly provided by Professor Timothy Close, University of California at Riverside. After three consecutive washes for 10 min each in TBS-Tween 20 (5%, v/v), primary antiserum was detected with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (Promega, Madison, Wis., U.S.A.). The secondary antiserum was used diluted at 1 : 10000 in 0.5% nonfat dried milk in TBS for 90 min. The resulting protein-antibody complex was visualized with 10 ml of AP buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 66 μl of nitroblue tetrazolium and 33 μl of 5-bromo-4-chloro-3-indolylphosphate (Promega). The specificity of the dehydrin detection was confirmed by blocking the primary antiserum for 30 min with an equal volume of the K segment peptide salt (5 mg/ml) prior to the first incubation of the membranes.

Immunogold electron microscopy

Cotyledons and axes were processed separately. Embryos were fixed in 2.5% glutaraldehyde in 0.1 M phosphate, pH 7.2, for 12 h at 4 °C and then sectioned into two parts. Fixed tissue was then washed three times at 30 min intervals in 0.1 M phosphate, pH 7.2, and dehydrated in a graded ethanol series. Dehydrated tissue was embedded in London Resin White resin (17411; Polysciences, Inc., Warrington, Pa., U.S.A.) following an established procedure (Harris et al. 1994).

Ultrathin sections were picked up on Formvar-coated 100-mesh nickel grids (7550N, Polysciences) and incubated in blocking reagent (5% nonfat dried milk in 0.1 M TBS, pH 7.5) for 90 min. They were then incubated overnight at 4 °C in a purified anti-dehydrin antiserum diluted 1 : 50 in 0.5% nonfat dried milk in TBS buffer. After three washes with TBS plus 1% nonfat dried milk, sections were incubated for 1 h with goat anti-rabbit colloidal gold antiserum (10 nm diameter particles, G-7402; Sigma Chemical Co., St. Louis, Mo., U.S.A.) diluted with TBS buffer (1 : 50), and subsequently washed 3 times with TBS plus 1% nonfat dried milk and distilled water. Finally, they were stained with 1% (w/w, aqueous) uranyl acetate (21447, Polysciences) followed by 1% lead citrate (0785, Polysciences), and examined with a Zeiss EM109T transmission electron microscope. Control sections were incubated as before, excluding the purified anti-dehydrin antiserum, and confirmed by blocking the primary antiserum for 60 min with an equal volume of the K segment peptide salt (5 mg/ml) prior to the first incubation.

Results and discussion

Unlike orthodox seeds, recalcitrant seeds are sensitive to desiccation in the mature state, thus providing – when pre-

sent – an interesting system to study dehydrins (Kermode 1997). Recently, recalcitrance or desiccation tolerance has been viewed more quantitatively, rather than from the previous "all or nothing" stance (Walters 2000). Under this new perspective, *E. edulis*, in which mature embryo tissues are in an extremely desiccation-sensitive state, is categorized as an extremely recalcitrant species (Panza et al. 2004).

The presence of dehydrins in this species was studied using a rabbit antiserum raised against the K segment common to dehydrins (Close et al. 1993) by Western blot analysis after 10% SDS-PAGE. As dehydrins often exhibit organ and tissue specificity (Kermode 1997), endosperm and embryos were studied separately. Our results evidenced the presence of three major dehydrin bands at 16, 18, and 24 kDa that were common for both tissues (Fig. 1, lanes B and C). As a control for the specificity of dehydrin detection, we incubated a duplicate blot with primary antiserum that had been previously blocked by incubation with purified K segment peptide. In this case, no dehydrin bands were observed (data not shown). Wheat embryo tissue samples were included as a positive control because wheat seeds present orthodox behaviour and because this species belongs to the tribe Triticeae of the family Poaceae, in which the presence of dehydrins is well documented (Campbell and Close 1997, Zhu et al. 2000) (Fig. 1, lane D). The use of wheat as a control emphasizes the strong conservation of this amino acid sequence motif among species with very different storage behaviour. In addition, the comparative analysis also evidenced differences between both species regarding dehydrin molecular mass and diversity (Fig. 1, lanes B–D). In fact, wheat embryo bands were highly diverse compared with the three bands present in *E. edulis* embryos, suggesting differences between these species. Noticeably, the 24 kDa dehydrin band is not detected for *E. edulis* by Western blot after 15% SDS-PAGE (data not shown). A negatively stained band appears at the same position after immunodetection. This could be due to the presence of a very abundant protein (evidenced by a bright Ponceau S staining after electroblotting) which could impede access to, and thus the detection of, the dehydrin by the specific antiserum. In contrast, the 24 kDa reactive band and the hindering protein are better resolved in 10% gels, so the masking does not occur. Since desiccation tolerance is a quantitative feature (Vertucci and Farrant 1995), the number of dehydrins may be one of the factors affecting tolerance levels. The question arises as to whether the desiccation sensitivity of the recalcitrant species *E. edulis* is at least partially the result of an insufficient accumulation of dehydrins or whether other aspects (inability to produce dehydrins specifically associated with desiccation tolerance) may also be involved.

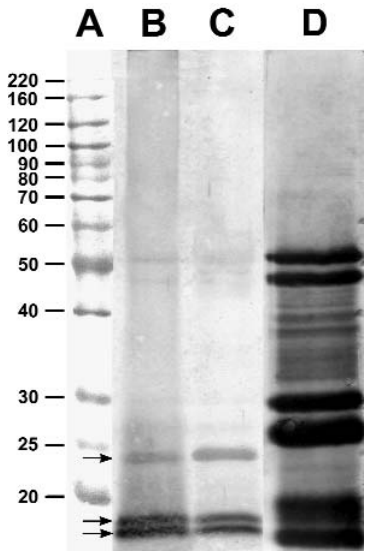

Fig. 1. Western blot analysis of dehydrin-related proteins present in mature seeds of *E. edulis* and *Triticum aestivum* cv. Chinese Spring. *A* Benchmark molecular weight standards. *B Euterpe edulis* endosperm heat-stable proteins. *C Euterpe edulis* embryo heat-stable proteins. *D* Wheat embryo heat-stable proteins. Arrows indicate *E. edulis* seed dehydrin bands of approximately 16, 18, and 24 kDa. The first antiserum was a purified anti-dehydrin antiserum directed against the K segment common to all dehydrins at a 1 : 1000 dilution. The second antiserum was an alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G at a 1 : 10000 dilution

Oliver and Bewley (1997) suggest that desiccation tolerance, an ancestral feature, has evolved in vegetative tissue at least twelve times, eight of these within angiosperms. Von Teichman and van Wyk (1994) propose that desiccation tolerance in seeds is likely subjected to strong homoplasy, i.e., parallelism, convergence, and character state reversals, making it difficult to determine whether the orthodox or recalcitrant habit is the most ancestral for a particular taxon. Despite these reservations, the authors consider seed recalcitrance to be an ancestral condition and suggest that desiccation tolerance may have evolved independently a number of times (Pammenter and Berjak 1999). In the palm family, constituted by recalcitrant, orthodox, and intermediate species (Hong et al. 1996), this subject awaits further investigations.

To date, dehydrins have not been detected in seeds of other palm species; consequently, we were unable to make evolutionary inferences within this family. In recalcitrant species from other families, an 18 kDa dehydrin has been detected in axes of *Aesculus hippocastanum* embryos and a 16 kDa dehydrin has been detected in *Barringtonia*

racemosa following axis drying (Farrant et al. 1996). A 24 kDa dehydrin has also been detected in *Zizania palustris*, a cold temperate aquatic grass with seeds exhibiting intermediate characteristics between recalcitrance and orthodoxy (Bradford and Chandler 1992). *Aesculus hippocastanum* and *Barringtonia racemosa* are both dicotyledons (Hippocastanaceae and Lecythidaceae, respectively) and *Zizania palustris* and *E. edulis* are monocotyledons (Poaceae and Arecaceae, respectively), and all of them are phylogenetically distant. The evident lack of an evolutionary pattern may be due to the low number of species in which dehydrins have been studied.

In order to determine the subcellular localization of dehydrins, sections of cotyledons, axis, and endosperm tissues were studied by immunogold electron microscopy and compared with results obtained by Houde et al. (1995) in wheat seedlings. In agreement with the immunolocalization in wheat, dehydrins were present in the cytoplasm and associated with chromatin in this study, but not with any organelles or membranes (Fig. 2A, B). *Euterpe edulis* embryo and endosperm tissue sections incubated with goat anti-rabbit colloidal gold-labelled immune serum without the purified anti-dehydrin antibody were free of labelling (Fig. 2C, D). In dehydrated cotton and maize embryos, dehydrins have been detected in both the cytoplasm and nuclei (Roberts et al. 1993, Asghar et al. 1994, Godoy et al. 1994, Close 1996, Egerton-Waburton et al. 1997, Colmenero-Flores et al. 1999). Although the function of dehydrins remains unknown, it is hypothesized that they stabilize membranes and macromolecules during cellular dehydration (Ismail et al. 1999, Karlson et al. 2003). In the highly hydrated *E. edulis* embryo cells,

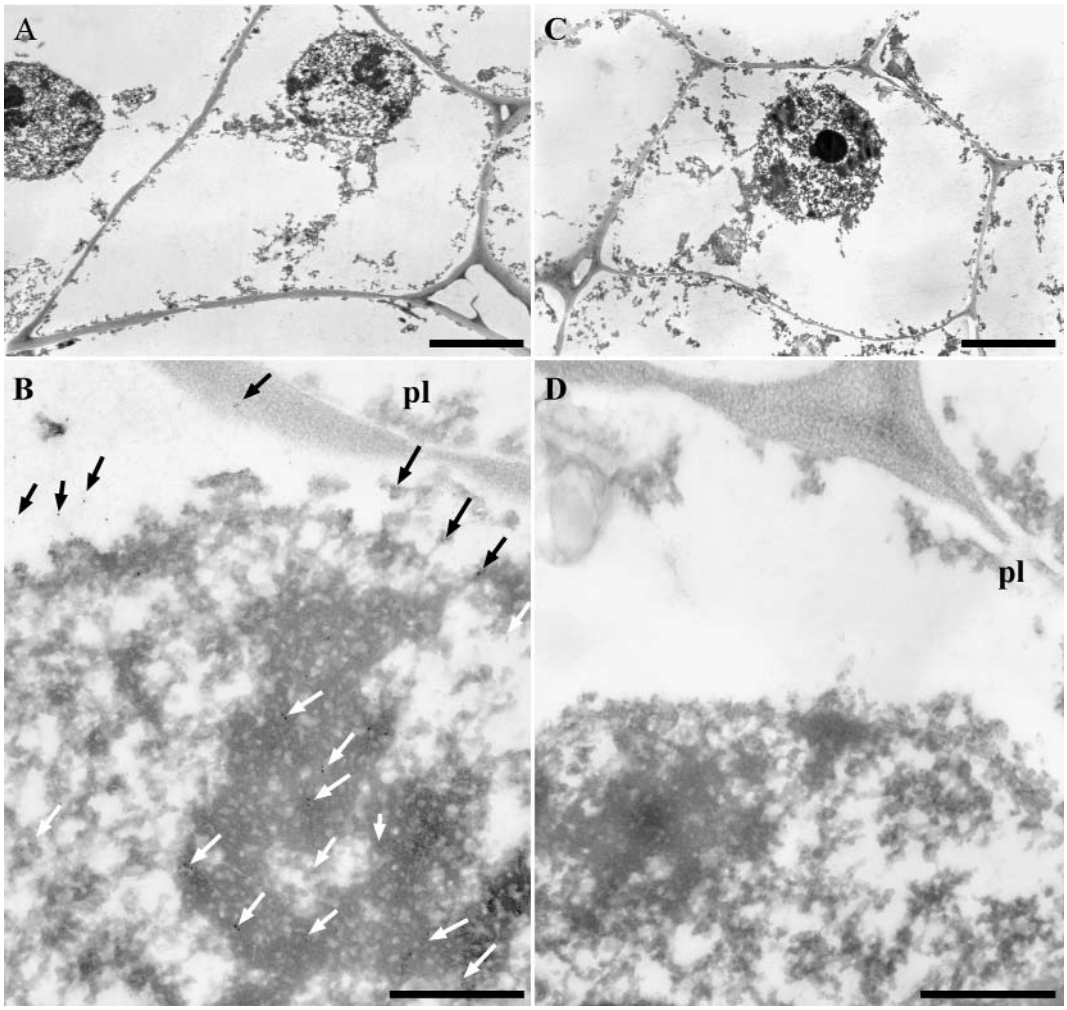

Fig. 2 A–D. Ultrathin sections of shoot apical meristem tissue of *E. edulis* embryos examined by transmission electron microscopy after immunolabelling. **A** Section labelled with goat anti-rabbit colloidal gold antiserum after incubation in a 1 : 50 diluted rabbit anti-dehydrin antiserum directed against the K segment common to dehydrins; **B** detail of **A**. **C** Control section incubated as the section shown in panel A but without the purified anti-dehydrin antiserum; **D** detail of **C**. *pl* Plasmodesmata; arrows indicate gold labelling. Bars: A and C, 3 μm; B and D, 0.5 μm

the role of dehydrins remains intriguing and warrants further investigation.

At present, much work remains to be done in order to precisely define the role of dehydrins in *E. edulis* seeds. According to Blackman et al. (1991) and Bradford and Chandler (1992), dehydrins are essential but not sufficient for the development of seed desiccation tolerance. Additional features, including tissue dedifferentiation, lipid, protein, and sugar accumulation, membrane phase transition, water replacement, and glassy state formation (Sun and Leopold 1997), are involved in anhydrobiosis. Our group previously reported that none of these additional characteristics occur in mature *E. edulis* embryos (Panza et al. 2004). It is now evident that dehydrins, at least in this species, are not sufficient to confer desiccation tolerance.

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