Protoplasma (2013) 250:911–918
DOI 10.1007/s00709-012-0474-7

ORIGINAL ARTICLE

Immunoanalysis of dehydrins in *Araucaria angustifolia* embryos

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Received: 20 November 2012 / Accepted: 10 December 2012 / Published online: 21 December 2012
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Abstract The aim of this study was to describe the dehydrin content of mature *Araucaria angustifolia* embryos, a species of endangered and economically important conifers, native to southern Brazil, northeastern Argentina, and eastern Paraguay. The *A. angustifolia* seeds have been categorized as recalcitrant. Dehydrins were studied by western blot analysis and in situ immunolocalization microscopy using antibodies raised against the K segment, a highly conserved lysine-rich 15-amino acid sequence extensively used to recognize proteins immunologically related to the dehydrin family. Western blot analysis of the heat-stable protein fraction, as estimated by 15 % SDS-PAGE, revealed three main bands of approximately 20-, 26-, and 29-kDa; when 17.5 % SDS-PAGE was used, each band resolved into two other bands. Two thermosensitive dehydrin bands of around 16 and 35 kDa were common to the axis and cotyledons, and another thermosensitive band, with molecular mass of approximately 10 kDa, was present in the cotyledons only. Following alkaline phosphatase (AP) treatment, a gel mobility shift was detected for each one of the four main bands that can be due to phosphorylation. Dehydrins were detected in all axis and cotyledon tissues using in situ immunolocalization microscopy. At the subcellular level, dehydrins were immunolocalized in the nuclei, protein bodies, and microbodies. In the nucleus, dehydrins were found to be associated with chromatin. We concluded that the gel mobility shift for the four main bands (probably due to phosphorylation), the presence of thermosensitive bands, and the specific localizations in nuclei and protein bodies provide key starting points to understand the function of dehydrins in the embryo cells of this species.

Keywords *Araucaria angustifolia* seed · Dehydrin western blot analysis · Dehydrin in situ immunolocalization · Phosphorylated dehydrins · Dehydrin subcellular localization · Thermosensitive dehydrins

Handling Editor: Friedrich W. Bentrup

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Introduction

Araucaria angustifolia (Bert.) O. Kuntze is an endangered conifer of economic importance, native to southern Brazil (commonly found in the states of Paraná, Santa Catarina, Rio Grande do Sul, and São Paulo), northeastern Argentina (Misiones and Corrientes), and eastern Paraguay (Alto Paraná) (Fig. 1), growing in low mountains at altitudes of 500–1,800 m. Today, the species, reduced to 1–2 % of its original area, faces the threat of extinction (Guerra et al. 2000).

Fig. 1 Natural geographical distribution of *Araucaria angustifolia* (Bert.) O. Kuntze. The species is native to southern Brazil, northeastern Argentina, and eastern Paraguay

A. angustifolia is considered recalcitrant because its intact seeds deteriorate rapidly under different conditions (Hong et al. 1996; Panza et al. 2002) and tolerate little subsequent desiccation. Actually, germination is reduced when moisture content falls below 37 %, while a desiccation level of 25 % leads to complete loss of germinability (Tompsett 1984). The embryo tissues of mature *A. angustifolia* seeds appear to mainly store lipids and starch, but their cells exhibit features that indicate a strategy of continuous development without the interposition of a dry state. In fact, embryo cells contain protein vacuoles very rich in water (Panza et al. 2002). Gametophytes store starch (the most conspicuous reserve observed) as well as protein and lipid bodies (Panza et al. 2002).

Late embryogenesis abundant (LEA) proteins are originally reported in cotton (*Gossypium hirsutum* L.) embryos by Dure et al. (1981). They are subsequently found in the seeds of many other plants and also in vegetative organs, especially under stress conditions such as cold, drought, or high salinity (Ingram and Bartels 1996; Rorat 2006; Battaglia et al. 2008; Burrieza et al. 2012). Plant LEA proteins are separated into different groups according to the occurrence of different amino acid sequences (Battaglia et al. 2008) and dehydrins constitute the second group of LEA proteins (Group 2, originally called D-11). These proteins are hydrophilic, thermostable, and contain the lysine-rich repeat K segment (EKKGIMDKIKEKLPG), which has been proposed to form an amphipathic helix (Close 1996, 1997). This structure allows both hydrophilic and hydrophobic interactions to stabilize proteins in water stress environments (Dure 1993). The S segment, a cluster of eight

serine residues, is present in many, but not all, members of the dehydrin family; this segment can undergo phosphorylation by the casein kinase 2 (CK2) as shown by Plana et al. (1991) and Riera et al. (2004) in RAB 17 of maize as well as Alsheikh et al. (2003) in ERD14 of *Arabidopsis*. Another conserved domain present in many dehydrins is the Y segment, which, with the consensus sequence DEYGNP, shares some similarities with the nucleotide-binding site of plant and bacteria chaperones (Close 1996).

Dehydrins accumulate in seeds when the embryos acquire desiccation tolerance (Battaglia et al. 2008). In this way, their synthesis in embryo tissues represents one of the programmed responses to dehydration that orthodox seeds undergo during the final stages of development. Dehydrins are absent in the embryos of mature recalcitrant seeds of the tropical wetland species *Avicennia marina* (Forssk.) Vierh., *Barringtonia racemosa* (L.) K. Spreng., *Bruguiera cylindrica* (L.) Bl., *B. exaristata* Ding Hou, *B. gymnorrhyza* (L.) Lamk., *Ceriops tagal* (Perr.) C.B., *Rhizophora apiculata* Lam., *R. mucronata* Lam., *R. stylosa* Griff., and *Trichilia dregeana* Sond. while present in the temperate species *Acer saccharinum* L (silver maple), *Acer pseudoplatanus* L. (sycamore), *Aesculus hippocastanum* L (horse chestnut), *A. angustifolia* (Bert.) O. Kuntze, *Camellia sinensis* (L.) O. Kuntze, *Castanea sativa* L. (European chestnut), *Euterpe edulis* Martius, *Poncirus trifoliata* (L.) Raf., *Porteresia coarctata* Tateoka, and *Quercus robur* L. (Bradford and Chandler 1992; Finch-Savage et al. 1994; Gee et al. 1994; Farrant et al. 1996; Han et al. 1997; Pammenter and Berjak 1999; Panza et al. 2007; Berjak and Pammenter 2008; Ismail et al. 2010).

In this study, we analyzed dehydrins in *A. angustifolia* (Bert.) O. Kuntze embryos using western blot and in situ immunolocalization with the primary antibody raised against the above-mentioned K segment. In order to detect thermosensitive bands, we used a fraction heated to 96 °C, as is the standard protocol, in addition to another nonheated fraction. This study is part of a comprehensive research project towards the conservation of *A. angustifolia* seeds.

Material and methods

Plant material

Seeds of *A. angustifolia* (Bert.) O. Kuntze were harvested at maturity from trees grown in the Experimental Station of Epagri, Lages-Santa Catarina, Brazil and the National Forest of Três Barras, Três Barras-Santa Catarina, Brazil. Collections were made in years 2010, 2011, and 2012. The seeds were immediately separated from the cones and dissected to extract embryos. Studies were made from all collections, and results presented in this study, both those of the western blot and in situ immunolocalization assays, are representative of the three seed harvests.

Extraction of embryo proteins and western blot assay

Embryos excised from seeds were cut to separate axis and cotyledon samples. Samples were lyophilized and ground to a powder in liquid nitrogen and then homogenized according to Burrieza et al. (2012). After centrifugation at 10,000*g* (two times), the pellet was discarded and the supernatant divided into two fractions. Fraction 1 was prepared following a modified version of the procedure described by Close and Lammers (1993): extracts were immersed in a bath at 96 °C for 10 min to be later cooled to room temperature and centrifuged at 10,000*g* (two times) for 10 min at 4 °C; pellet was discarded and the supernatant, kept. Fraction 2 was not heated so as to conserve thermosensitive proteins. Protein contents of both fractions were quantified using a Qubit Quantitation Platform Protein Assay Kit (Invitrogen, Carlsbad, CA, USA). Proteins were resolved by both 15 and 17.5 % SDS-PAGE (Laemmli 1970) at a constant voltage of 100 V for 120 min using a Mini-PROTEAN II Electrophoresis Cell II (Bio-Rad Laboratories, Hercules, CA, USA). Protein was loaded at 30 μg per lane and benchmark molecular weight standards (Invitrogen) were used. Following electrophoresis, the fractionated proteins were transferred onto a nitrocellulose membrane (Osmonics Inc., Minnetonka, MN, USA) at 100 V for 60 min using a Mini Trans-Blot Electrophoretic Transfer Cell II (Bio-Rad Laboratories, Hercules, CA, USA).

The western blot assays were carried out according to Burrieza et al. (2012). Protein molecular masses were calculated by the "gel perfect" program described by Bozzo and Retamal (1991) and Retamal et al. (1999).

Dephosphorylation

Fifty micrograms of embryo protein extract was diluted to 40 μl in 1× MULTI-CORE™ Buffer (Promega, Madison, WI, USA) in the presence or absence of 10 U of Thermostable Shrimp Alkaline Phosphatase (Promega) for 12 h at 37 °C. The western blot was performed like above. The relative mobility of each band was calculated using the "gel perfect" program above-mentioned.

Sample preparation for light and transmission electron microscopy

Excised embryos were transversally cut to obtain separate samples of axes: with root apical meristem or shoot apical meristem tissues, plus cotyledons. Samples were fixed using a mixture of 2 % paraformaldehyde and 0.5 % glutaraldehyde in 0.2 M phosphate buffer, pH 7.2; washed with 0.1 M

phosphate buffer, pH 7.2 for 30 min; dehydrated in a graded ethanol series and embedded in London Resin White resin (Polyscience, Inc., Warrington, PA, USA), according to Harris et al. (1995). Semithin (1 μm thick) and ultrathin sections were obtained for light microscopy (LM) and transmission electron microscopy (TEM), respectively, with an ultramicrotome (Reichert-Jung, Vienna, Austria) equipped with a diamond knife.

Immunolabeling for light microscopy

The tissue distribution of dehydrinlike proteins in embryo sections was immunolabeled according to Burrieza et al. (2012). Control sections were treated: (1) excluding the purified rabbit antidehydrin immune serum and (2) the primary antiserum was blocked with the recognized peptide sequence prior to the incubation step.

Immunolabeling for transmission electron microscopy

Ultrathin sections were mounted on Formvar-coated nickel grids (Polyscience, Inc., Warrington, PA, USA) for immunolabeling at the TEM level. Grids carrying sections were immunolabeled according to Carjuzaá et al. (2008). Finally, grids were counterstained with uranyl acetate followed by lead citrate and examined under a Zeiss EM 109 turbo (Zeiss, Wiesbaden, Germany) transmission electron microscope operating at an accelerating voltage of 90 kV. Control sections were treated: (1) excluding the purified rabbit antidehydrin immune serum and (2) the primary antiserum was blocked with the recognized peptide prior to the incubation step.

Results

Dehydrin western blot analysis in embryos

The dehydrin pattern of *A. angustifolia* embryos is shown in Fig. 2. The western blot analyses of the heat-stable protein fraction, as estimated by 17.5 % SDS-PAGE, revealed six major bands of 18, 20, 25, 26, 28, and 29 kDa in cotyledons and axis (Fig. 2a, lanes 3 and 4).

A thermosensitive band of around 16 kDa was detected in the cotyledons as well as in the axis (Fig. 2a, lanes 1, 2) and another thermosensitive band, of around 10 kDa, was detected in cotyledons only (lane 1). A third minor thermosensitive band of 35 kDa was also detected in cotyledon and axis (Fig. 2a, lanes 1 and 2). When 15 % SDS-PAGE was used, just four main bands were detected, i.e., 29, 26, 20, and 16 kDa, (Fig. 2b).

A gel mobility shift was revealed for the four main bands, following alkaline phosphatase (AP) treatment, which can be attributed to phosphorylation (Fig. 2b). As a control for the specificity of dehydrin detection, duplicate membranes were incubated with primary antiserum preblocked with the consensus K peptide. No bands were present in the control membranes, indicating that all bands detected are immunologically related to the dehydrin family (data not shown).

In situ immunolocalization of dehydrins in embryos

Western blot analysis was complemented by in situ immunolocalization of dehydrins in cotyledons and axis in order to establish the tissue and subcellular localization of these

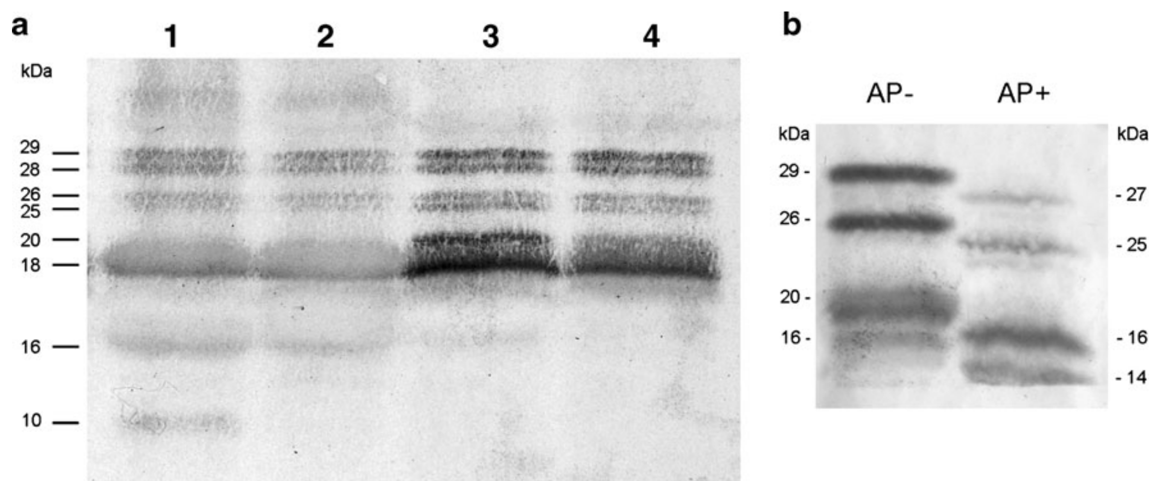

Fig. 2 Western blot analyses of dehydrinlike proteins present in *A. angustifolia* embryos. **a** Dehydrins were separated by 17.5 % SDS-PAGE and electroblotted onto nitrocellulose membranes. The first antiserum was a purified antidehydrin antiserum directed against the K domain present in all dehydrins. The second antiserum was an alkaline phosphatase-conjugated goat antirabbit immunoglobulin G. *Lanes 1 and 3* cotyledon, *2 and 4* axis, *1 and 2* total proteins, *3 and 4* from heat-stable fractions. **b** Proteins were diluted in buffer, without (−) or with (+) alkaline phosphatase (*AP*) treatment. Dehydrins were separated by 15 % SDS-PAGE, electroblotted onto nitrocellulose membranes, and revealed as above described for **a**. In both figures, protein molecular masses were calculated by the Gel Perfect Software. The figure is a representative of the seed collections

proteins. Dehydrins were detected in all axis and cotyledon tissues but accumulation was significantly greater in the shoot apical meristem tissue (Fig. 3). At the subcellular level, dehydrins were detected in nuclei and in the matrix of the protein bodies (Figs. 3, 4 and 5). In the nucleus, dehydrins were associated with chromatin (Figs. 3, 4 and 5). Labeling was also detected in the matrix of some

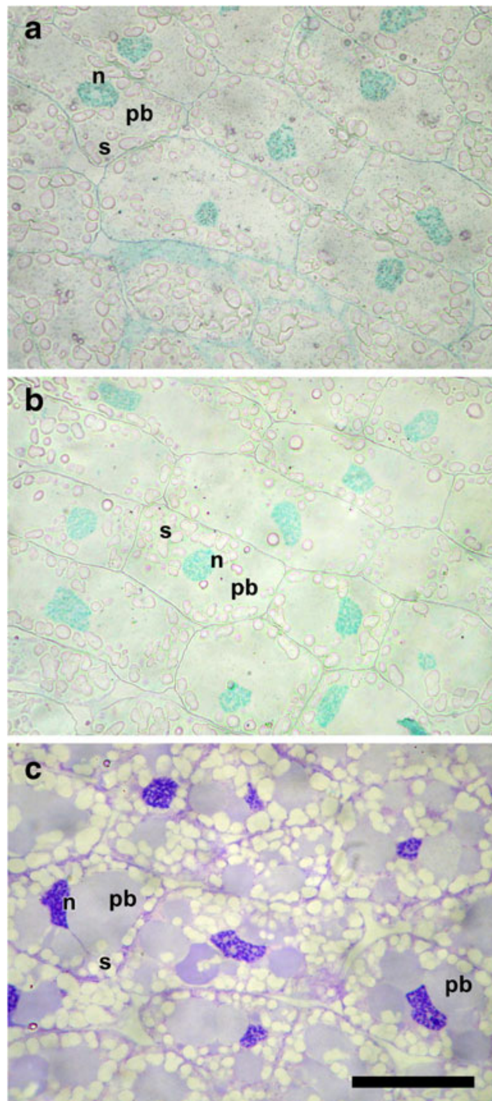

Fig. 3 **a**–**c** Dehydrin in situ immunolocalization using the K domain-specific antiserum in *Araucaria angustifolia* mature embryo axis tissue. **a** This section was incubated with purified rabbit antidehydrin primary antibody, then in colloidal gold-conjugated goat antiserum to rabbit immunoglobulins, and revealed by a silver enhancement kit until developing a brown-to-black stain in the immunoreactive areas. Labeling was mainly observed in nuclei, specifically in chromatin, and also in protein bodies. **b** Control section: this section had been incubated with primary antiserum, previously blocked with purified lysine-rich peptide derived from the K segment. In **a** and **b**, sections were contrasted with Fast Green. **c** An untreated section stained with toluidine blue. *n* nucleolus, *pb* protein body, *s* starch. Scale bars: 40 μm. The figure is a representative of at least five embryos from each harvest

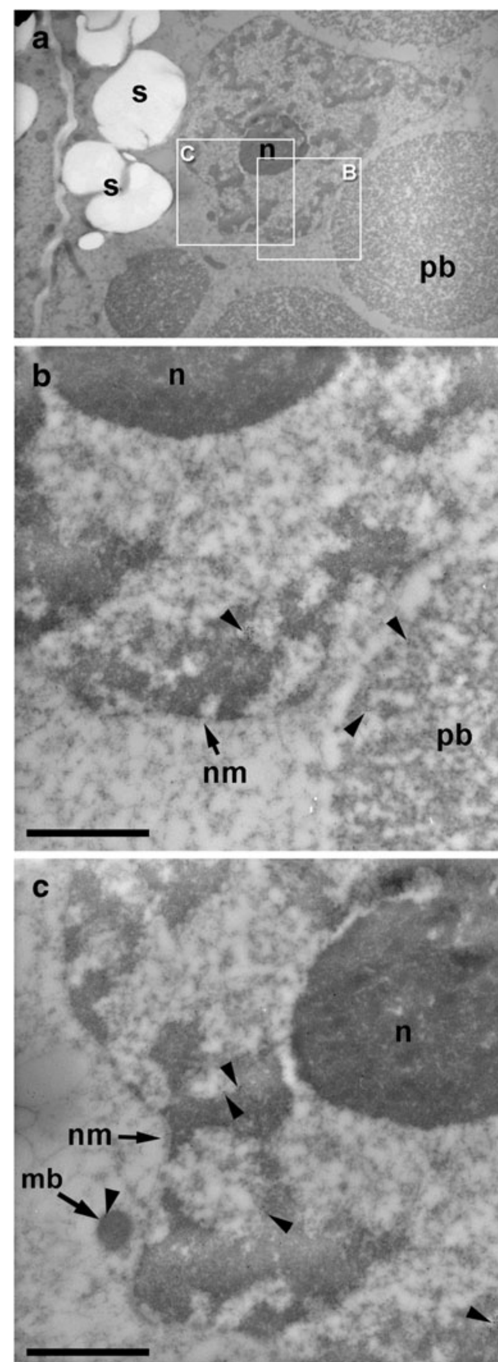

Fig. 4 **a** In a cell from shoot apical meristem of a mature *A. angustifolia* embryo, dehydrins are mainly associated with chromatin. Ultrathin section was incubated with the purified rabbit antidehydrin primary antibody in colloidal gold-conjugated goat antiserum to rabbit immunoglobulins. This section was observed by TEM. *Arrowheads* indicate colloidal gold particles (10 nm). Labeling was mainly observed in nuclei, specifically in chromatin and in the matrix of the protein bodies. **b**–**c** Details of **a**. In **c**, the matrix of a microbody, probably a peroxisome, was also labeled. *mb* microbody, *n* nucleolus, *nm* nuclear membrane, *pb* protein body, *s* starch. Scale bar: 1 μm. The figure is a representative of at least 20 cells from three different samples

microbodies, probably peroxisomes (Fig. 4c). No labels were found in control sections, i.e., sections that had not

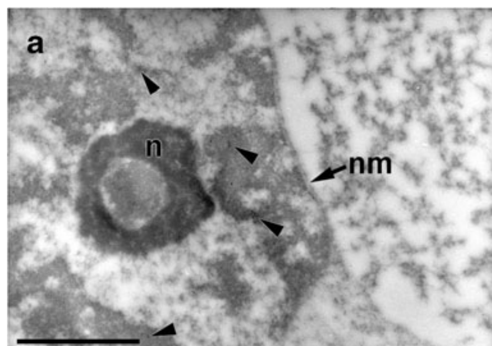

Fig. 5 In a cell from root apical meristem of a mature *A. angustifolia* embryo, dehydrins are associated with chromatin. This section was obtained and treated as in Fig. 4. *n* nucleolus, *nm* nuclear membrane. Scale bar: 1 μm. The figure is a representative of observations of at least 20 cells from three different samples

been incubated with the purified rabbit antidehydrin immune serum (not shown) and sections that had been incubated with primary antiserum previously blocked with purified lysine-rich peptide derived from the K segment common to all dehydrins (Fig. 3b).

Discussion

Western blot analysis for the detection of LEA proteins like dehydrins using 15 % SDS-PAGE and heat-stable protein fractions revealed three major bands of around 20, 26, and 29 kDa in axis and cotyledons; when 17.5 % SDS-PAGE was used, each one of those three bands resolved into other two bands. Two thermosensitive dehydrin bands of around 16 and 35 kDa were common to axis and cotyledons, and another one with a molecular mass of approximately 10 kDa, also thermosensitive, was present in the cotyledon only. Farrant et al. (1996) report three bands of 23, 26, and 28 kDa, but that study lacks an image of the western blot assay and does not include the acrylamide concentration of the gel. These authors do not mention where the seeds were collected nor how they were stored, which are both important because seeds of this species are recalcitrant and can degrade rapidly depending on their storage.

The discovery that thermosensitive dehydrins are constitutively expressed in seeds is almost unique. In the literature available, we only found three relevant references, two from studies on vegetative tissues: one on constitutive and heat-sensitive dehydrins in fucoid algae (Li et al. 1998) and the other on how three constitutive and heat-sensitive dehydrins accumulate in the mitochondria of wheat, barley, and maize seedling tissues (Borovskii et al. 2002). The only study where a heat-sensitive dehydrin was found in seeds was by Gumilevskaya and Azarkovich (2010) who found a band of 80 kDa in the mature recalcitrant seeds of horse chestnut (*A. hippocastanum* L.).

In *A. angustifolia* seeds, we found that dehydrins are immunolocated mainly in the nuclei of all embryo cells. Dehydrin labeling was reduced in cytosol but abundant in the matrix of protein bodies. Labeling was also detected in the matrix of some microbodies. The data in the literature obtained from different species indicate that most dehydrin proteins accumulate in vegetative tissues upon water deficit imposed by drought, low temperature, or salinity, although others preferentially respond to particular stress conditions: some dehydrins are strongly accumulated in response to low temperature treatments but not to drought or salinity (Rorat 2006). In fact, most quinoa embryo dehydrins are expressed independently of environmental conditions (Carjuzaá et al. 2008). Although seed dehydrins often exhibit tissue and organ specificity, immunolocalizations performed in cotton, maize, and quinoa seeds have shown that they are present in all embryo tissues (Ashgar et al. 1994; Danyluk et al. 1994; Close 1996; Carjuzaá et al. 2008; Burrieza et al. 2012). To date, dehydrin subcellular immunolocalization studies have only been carried out in the seeds of four species: maize, wheat, quinoa, and *E. edulis*. In maize, dehydrins are only present in the cytosol and nucleus (Ashgar et al. 1994; Goday et al. 1994; Egerton-Warburton et al. 1997; Jensen et al. 1998). In wheat, they are present in the nucleus (Brini et al. 2007), while in *E. edulis*, they are found in the cytoplasm and are associated with chromatin but not with organelles or membranes (Panza et al. 2007). Data on subcellular localization in nonseed tissues reveal that dehydrins are primarily located in the cytoplasm and nucleus but are also present in various other cell compartments, such as in the area of the plasma membrane (Danyluk et al. 1998), mitochondria (Borovskii et al. 2000), chloroplasts (Wisniewski et al. 1999), endoplasmic reticulum (Neven et al. 1993), vacuoles (Heyen et al. 2002), protein bodies (Rinne et al. 1999; Carjuzaá et al. 2008), and amyloplasts (Rinne et al. 1999).

The presence of dehydrins in nuclei not only indicates protection of the transcriptional machinery but also raises the question of how these proteins are targeted to the nucleus. In this regard, Battaglia et al. (2008) suggest that the transport of different types of dehydrins to the nucleus occurs via different nuclear localization pathways: on one hand, Plana et al. (1991) suggest that in maize embryo, the phosphorylated S segment and the RRKK sequence constitute nuclear localization signals, while, on the other hand, Riera et al. (2004) affirm that nuclear localization is independent of the phosphorylation state of the S segment and furthermore, that proteins lacking the S segment or RRKK motif are located in the nucleus.

The presence of dehydrins in the matrix of the protein bodies of *A. angustifolia* embryo tissues is not a novel finding, as they have been reported in both maize (Egerton-Warburton et al. 1997) and quinoa embryos (Carjuzaá et al. 2008). Since embryo protein reserves are consumed during germination, it

is conceivable that the major role of dehydrins stored in protein bodies is to help seedling withstand probable dehydration throughout germination.

The presence of dehydrin in the matrix of some microbodies, probably peroxisomes, of *A. angustifolia* embryo tissues, is a novel observation. In a study of nine *Araucaria* species, Tompsett (1984) distinguished three types of seeds according to storage behavior with *A. angustifolia* belonging to the recalcitrant category. In this regard, we consider that a comparative study of dehydrins among *Araucaria* species, including orthodox species, warrants further investigation.

Conclusion

The gel mobility shift for the four main bands (probably due to phosphorylation), the presence of thermosensitive bands, and the localizations in nuclei, specifically in chromatine and in the matrix of the protein bodies constitute a key starting point to understand the function of dehydrins in the embryo cells of this species.

Acknowledgments We would like to thank Timothy Close for supplying the antidehydrin antiserum and peptide salt. Funding for this work was provided by the Universidad de Buenos Aires (X125 to S.M.), Ministerio de Ciencia y Tecnología/Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (MINCyT/CAPES 08/10 to SM and MPG), and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC to MPG). For their fellowships, FLF thanks the CNPq and FAPESC and HPB, the Universidad de Buenos Aires.

Conflict of interest None.

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