

Multiple Subcellular Localizations of Dehydrin-like Proteins in the Embryonic Axes of Common Beech (*Fagus sylvatica* L.) Seeds During Maturation and Dry Storage

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Abstract The accumulation and localization of dehydrin-like proteins were analyzed in the embryonic axes of beech (*Fagus sylvatica* L.) seeds during maturation between the 12th and 19th week after flowering and in the dry state after 2 years of storage. Three dehydrin-like proteins were reported using an antibody specific to the K-segment, and their subcellular localization was investigated using an immunocytochemistry approach. All dehydrin-like proteins were phosphorylated, and two, with molecular masses of 26 and 44 kDa, accumulated in mature seeds. During seed maturation, dehydrin-like proteins were frequently detected inside the round or segmented nuclei, along the cell plasma membrane and associated with small vesicles. Moreover, they were found to be attached to the mitochondrial and amyloplast envelopes. These proteins were also located in the cytosol and were associated with membrane structures throughout the cytoplasm. Conversely, in the embryonic axes of stored seeds, fewer specific locations were found; dehydrin-like proteins were associated mostly with amyloplasts, and were detected to a lesser extent in the nuclei, vacuoles, and other cytoplasmic structures. Bioinformatic tools were used to predict the putative beech dehydrin sites that interact with DNA, proteins, and lipid membranes to

highlight the reported subcellular locations. Here, we present and discuss the possible roles of dehydrin-like proteins in seeds with respect to their subcellular localizations.

Keywords Beech · Dehydrin · Seed development · Seed storage · Subcellular localization

Introduction

Common beech (*Fagus sylvatica* L.) is native throughout almost all of Europe and produces seeds in large intervals, which were classified at first into the *intermediate* category (Bonner 1990; Gosling 1991) and then into the *orthodox* category (Poulsen 1993; Poulsen and Kundsén 1999). During development, beech seeds become desiccation tolerant beginning at the 16th week after flowering (WAF) (Kalemba and others 2009) like all other *orthodox* type seeds (Roberts 1973). Beech seeds were finally classified into the *intermediate* category (León-Lobos and Ellis 2002) due to their reduced longevity during storage (León-Lobos and Ellis 2002; Pukacka and Ratajczak 2007; Kalemba and Pukacka 2014); the seeds cannot survive dehydration below those in equilibrium with approximately 40–50 % relative humidity (Ellis and others 1990).

Late embryogenesis abundant (LEA) proteins, including dehydrins, are hydrophilic molecules accumulated during development and water stress (reviewed in Rorat 2006; Battaglia and others 2008; Hara 2010; Battaglia and Covarrubias 2013). Dehydrins, group 2 of the LEA proteins, are characterized by highly conserved sequences, including a lysine-rich K-segment, a polyserine S-segment, a Y-segment, and a Φ -segment (Close 1996). The K-segment uniquely contains the consensus amino acid sequence

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EKKGIMDKIKELPG, which is present in all types of dehydrins (Close 1997). Despite the conserved dehydrin sequences, they appear to be intrinsically disordered proteins that may exert chaperone activity through an entropy transfer mechanism (Tompa and Csermely 2004). Structural disorder confers the ability to carry out more than one function (Kovacs and others 2008). Dehydrins are synthesized during seed development as an element of the embryogenesis program, and their accumulation is related to the acquisition of desiccation tolerance (Close 1996; Kalemba and others 2009). The structural and functional characteristics of LEA proteins are being investigated to understand their role during the adaptive response to water deficit in plants (Rorat 2006; Battaglia and others 2008; Hara 2010; Battaglia and Covarrubias 2013). LEA proteins can prevent protein aggregation (Goyal and others 2005) and protect cellular macromolecules and membranes under cellular dehydration (Rorat 2006; Battaglia and others 2008; Hara 2010; Battaglia and Covarrubias 2013). The proteins may exhibit radical scavenging activity (Hara and others 2004) and act as membrane and protein stabilizers during water stress either by direct interaction or by acting as molecular shields (Tunnacliffe and Wise 2007). Dehydrins and dehydrin-related proteins were detected in many plant seeds, including the *orthodox* woody plant seeds (Greggains and others 2000; Kalemba and Pukacka 2012), *intermediate* seeds (Kalemba and Pukacka 2008), and many *recalcitrant* woody plant seeds (Finch-Savage and others 1994; Sunderlikova and others 2009; Panza and others 2007; Vornam and others 2011; Farias-Soares and others 2013). Dehydrins present with different tissue localization during optimal growth conditions. Immunohistochemical localization of dehydrins demonstrated tissue and cell-type specificity in unstressed *Arabidopsis thaliana* plants, which suggested functional specialization for members of the dehydrin family (Nylander and others 2001). RAB1 localization in provascular tissue of the cotyledons and the radicle was reported in *Arabidopsis* seeds (Nylander and others 2001) and in maturing pea seeds (Garnczarska and others 2008). Dehydrins were also detected in wheat crown tissues (Houde and others 1995), and bark tissues of current-year-shoots of peach (Wisniewski and others 1999). Interestingly, subcellular investigations revealed that dehydrins were reported in chloroplasts (Mueller and others 2003), mitochondria (Borovskii and others 2005), endoplasmic reticulum (Neven and others 1993), amyloplasts (Rinne and others 1999), and in the plasma membrane vicinity (Danyluk and others 1998) of seedlings or mature plant organs. In seeds, dehydrins have been immunolocalized at the subcellular level to the cytoplasm and nucleus (Asgar and others 1994; Close 1996; Egerton-Warburton and others 1997; Panza and others 2007; Carjuzaa and others 2008; Lin and

others 2012). Dehydrins have also been found associated with cytoskeletal elements, the plasma membrane, the matrix of protein bodies, mitochondria, rough endoplasmic reticulum cisternae and proplastid membranes in quinoa (Carjuzaa and others 2008), and microbodies of *Araucaria angustifolia* (Bert.) embryos (Farias-Soares and others 2013).

To address the question of the potential roles of dehydrins and dehydrin-like proteins in beech seeds, we analyzed their subcellular localization during the seed maturation stage and in dry, stored seeds. The functions of dehydrin proteins are largely unknown. Therefore, we decided to investigate their potential role in cell protection during natural dehydration during seed development and seed storage.

Materials and Methods

Material Collection

Common beech (*F. sylvatica* L.) seeds were collected in the cropping year 2009 in Kórnik Arboretum (Western Poland, 52°24'37"N 17°09'51"E) from a single tree every 7 or 10 days beginning from the 12th WAF up to the 19th WAF. Nineteenth WAF seeds were considered as fully mature because it was the time when the shedding began. Seeds intended for protein isolation were prepared for storage at -80°C . The seed coats were removed, and embryonic axes were isolated from cotyledons because all analyses were performed using embryonic axes only. Preparation of the maturing seeds intended for immunocytochemistry was initiated immediately after harvesting. The water content (dry weight basis) was determined at each seed collection. One seedlot of beech seeds from the same tree was stored at -10°C in closed plastic boxes at 8–9 % water content for 2 years, and these seeds were then used for the immunocytochemistry analyses.

Protein Extraction and Electrophoresis

Embryonic axes were ground to a powder in liquid nitrogen. To obtain soluble proteins, the dried powder was homogenized at 4°C in a 1:2 (w:v) extraction buffer containing 20 mM Tris-HCl, pH 7.5, 5 % glycerol, 10 mM DTT, and 1 % protease inhibitor cocktail (Sigma-Aldrich, Poland) and 1.5 % polyvinylpyrrolidone (Sigma-Aldrich, Poland); the samples were further centrifuged ($20,000\times g$ at 4°C for 20 min). Protein concentration was measured according to the Bradford (1976) method using bovine albumin as a standard. Proteins were resolved with 12 % SDS-PAGE (Laemmli 1970) using the Mini-PROTEAN[®] Tetra Cell (Bio-Rad) system and were stained with silver nitrate

according to the method of Sinha and others (2001). To visualize phosphoproteins, gels were stained with Pro-Q[®] Diamond (Invitrogen) according to the standard manufacturer's protocol. The images were documented on a fluorescence imager (Ettan DIGE Imager, GE Healthcare) with excitation/emission settings at 555/580 nm.

Alkaline Phosphatase Treatment

Alkaline phosphatase treatment was performed on the soluble protein fractions extracted from the embryonic axes of beech seeds stored for 2 years. For enzymatic dephosphorylation, 100 mg of protein extract was treated with 4 units of calf intestine alkaline phosphatase (Sigma-Aldrich, Poland). The reaction was performed in CIP buffer with the enzyme at 37 °C for 16 h.

Western Blot

The protein extract (12.5 µg) was loaded onto a gel. The amount was calculated based on the calibration curve data in which the relationship between Western blot band intensities and the amount of the loaded protein was linear (correlation coefficient, $r = 0.98$). The fractionated proteins were transferred onto a polyvinylidene fluoride membrane (ImmobilonTM-P, Millipore) at 350 mA for 1 h and were then blocked and incubated with a rabbit primary antibody (dilution 1:1,000) raised against the dehydrin consensus K-segment (Close and others 1993). The secondary antibody was conjugated with alkaline phosphatase (Sigma-Aldrich, Poland) and was used in 1:10,000 dilutions. Protein bands were visualized on the membrane by reaction with an alkaline phosphate substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma-Aldrich). Control immunoassays were also performed with the dehydrin K-segment specific antibody blocked with the synthetic peptide TGEKKGIMDKIKEKLPQGH (Close and others 1993).

Densitometry Analysis

Western blot images, in triplicate, were analyzed using the UviBand (UviTec) program with a Fire Reader Gel Documentation System. Densitometry image analysis was based on the digitalization of the image in pixels with intensity coded on a scale of 256 gray levels. The density of a spot was calculated from its volume (V), which is a sum of all 3D intensities (I). The data were presented in relative units obtained from $V = \sum n_i I$ and from the number of pixels inside the area of the spot.

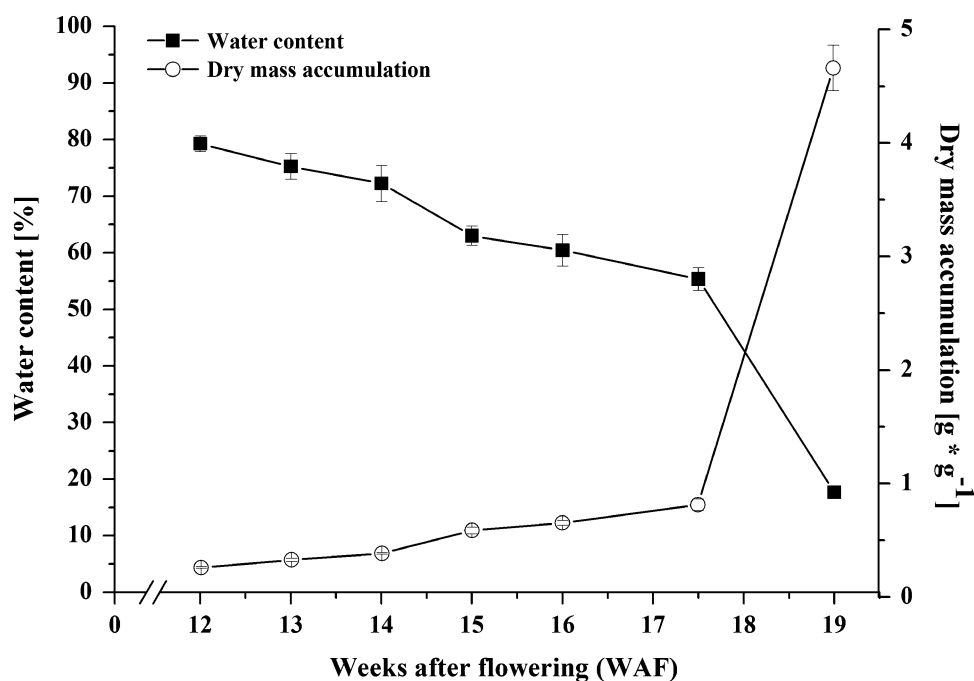
Immunocytochemistry

To determine localization of the dehydrin-like proteins, the samples were treated according to the procedure described by Bagniewska-Zadworna (2008) with small modifications. The material was fixed in 0.5 % (v/v) glutaraldehyde and 4 % formaldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 2 h at 4 °C. After dehydrating in a graded ethanol series, material was embedded in LR White resin (Sigma) and sectioned using an ultramicrotome Reichert Ultracut S (Leica). Moreover, before the experiment, osmium tetroxide post-fixation and embedding in the Spurr's resin were also used to verify the ultrastructure of each cell compartment and tissues of the material analyzed, according to the procedure described by Bagniewska-Zadworna and others (2010). The analyzed cells came from the root-hypocotyl axis (up to the distance of 1.5 mm from radical tip), because we demonstrated that the accumulating ROS that spread from the root cap to the root apical meristem in embryonic axes of *F. sylvatica* seeds are key factors that affect the success of long-term storage (unpublished data) as well as *Brassica napus* root is the most susceptible tissue to water deficit stress soon after germination (Bagniewska-Zadworna 2008). Ultrathin sections on nickel grids were blocked in PBS/2 % skim milk for 15 min at room temperature and were then incubated on a drop of primary antibody (raised against the dehydrin consensus K-segment (Close and others 1993), 1:100) in PBS/2 % skim milk overnight at 4 °C in a humid chamber. After careful washing in PBS, the sections were incubated with 15 nm gold-labeled goat anti-rabbit secondary antibody diluted 1:20 in PBS/1 % skim milk at 37 °C for 2 h. Subsequently, the sections were washed with PBS and rinsed with distilled water. The experiment was also performed without primary antibodies to assess the specificity of labeling. Sections were examined with a JEM 1200 EX II (Jeol, Tokyo, Japan) transmission electron microscope. On average, 5 different grids from each experimental variant were investigated.

Statistical Analysis

Data are presented as the mean \pm standard deviation of 3 biological replicates. The significant differences between particular parameters were tested using a correlation coefficient analysis. The significance among the means of components (between-group component and within-group component) was verified by F test at $P < 0.05$, and significantly different values are marked with different letters in the graph.

Fig. 1 The water content and dry mass accumulation of embryonic axes of beech seeds during maturation time between the 12th and 19th week after flowering (WAF). Water content is calculated as the dry weight basis and presented as a percentage. Dry mass accumulation is presented with [$\text{g} \cdot \text{g}^{-1}$] units referring to dry weight [g] divided by H_2O [g]. Presented data are the means of three independent replicates, \pm SD



Bioinformatic Tools

The ability of dehydrin proteins to interact with DNA was investigated using the DISis (<http://cubic.bioc.columbia.edu/services/disis>; Ofran and others 2007) and DP-Bind (<http://lcg.rit.albany.edu/dp-bind/>; Hwang and others 2007) programs using the dehydrin protein sequence named dehydrin/response ABA protein from *F. sylvatica* with accession number CAE54590.1 (Jiménez and others 2008) as the query. The Profisis (ISIS) program was run at the PredictProtein server (<https://www.predictprotein.org/>; Ofran and Rost 2006) to predict possible sites of dehydrin-protein interactions. Monte Carlo simulations of helical peptides in association with lipid membranes were made using the MCPep server (<http://bental.tau.ac.il/MCPep/>; Gautier and others 2008).

Results

Water Content

The embryonic axes of beech (*F. sylvatica* L.) seeds were analyzed during their maturation between the 12th and 19th WAF, and the water content (dry weight basis) and dry weight were determined (Fig. 1). The water content at the 12th WAF was high and reached 80%; however, after 16 weeks of maturation, the water content decreased to approximately 18% (at 19th WAF) because of the dry mass accumulation.

Expression and Characterization of Dehydrin-like Proteins

Three dehydrin-like proteins were detected in protein extracts of common beech (*F. sylvatica* L.) embryonic axes during seed maturation beginning from the 12th up to the 19th WAF (Fig. 2a). Soluble protein extracts were used for comparison with the immunocytochemistry data, which were generated using whole embryonic axes containing all protein fractions. However, in previous analyses, we had determined that in stored beech seeds, the 26 and 44 kDa dehydrins were heat-stable, and the 37 kDa protein was more sensitive to high temperatures (Kalemba and Pukacka 2008). The 44 kDa protein was the most abundant dehydrin during seed development and was clearly detectable since 14th WAF. Then, at the 16th WAF, the 44 kDa protein content increased, and progressive accumulation was observed until the end of maturation (Fig. 2a). The protein band corresponding to the 44 kDa dehydrin was analyzed densitometrically (Fig. 2b) and was compared with the water content data (Fig. 1). The correlation coefficient ($r = -0.9046$) indicated a statistically significant relationship between the 44 kDa dehydrin level and water content in embryonic axes, while the P value equaled 0.0051 indicating that the water-related expression of the 44 kDa dehydrin is important for the seed developmental process. The 26 kDa dehydrin protein was noted at the end of the seed maturation time and gave a weak protein band (Fig. 2a). Conversely, the dehydrin-like protein with an approximately 37 kDa molecular weight was present throughout the time of maturation and its concentration remained relatively unchanged. Interestingly, we

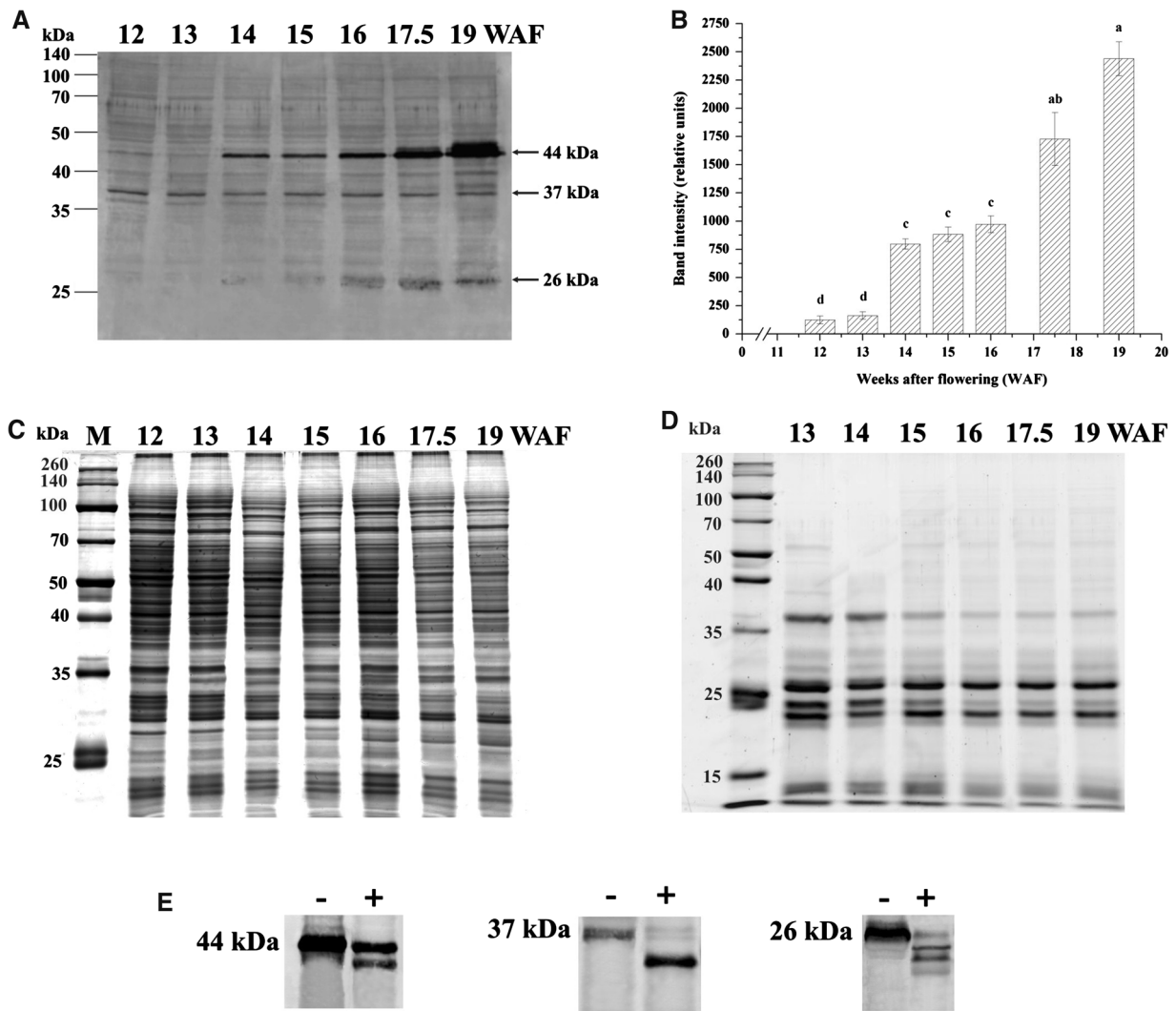


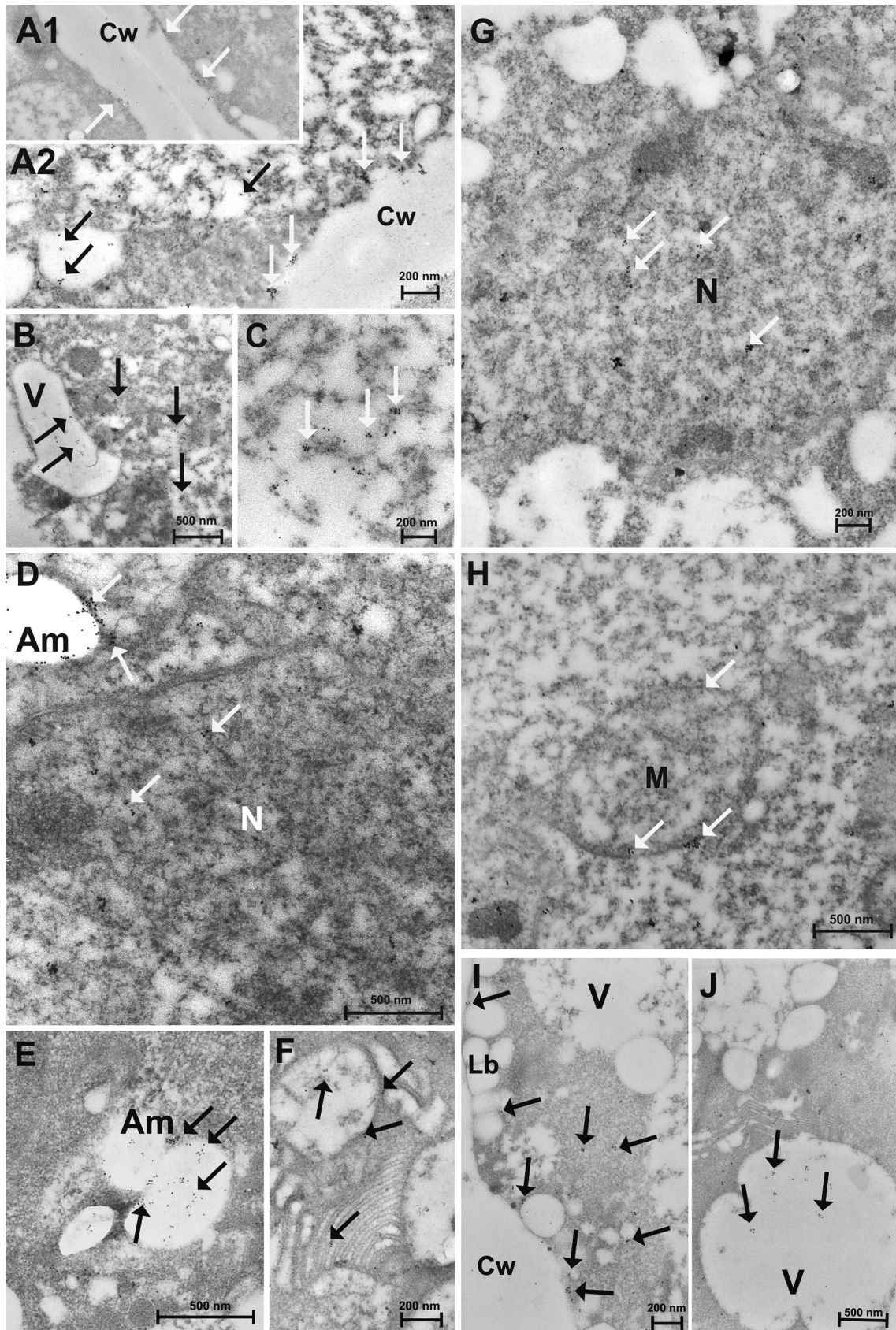
Fig. 2 Detection of dehydrin-like proteins in the embryonic axes of beech (*Fagus sylvatica* L.) seeds during maturation using an antibody specific to the K-segment (a). The dehydrins and dehydrin-like proteins were compared to soluble protein extracts resolved using a 12 % polyacrylamide gel and stained with silver (c). Intensities of the 44 kDa dehydrin signal are shown in the graph (b). Phosphorylated soluble proteins were visualized with Pro-Q[®] Diamond staining (d).

Phosphorylation was examined by enzymatic reaction with phosphatase (+) and then compared to non treated protein extracts (–) using the Western blot method (e), a representative dephosphorylation experiment is shown. Three independent biological repetitions were performed for each analysis. The Spectra Multicolor Broad Range Protein Ladder (Fermentas) molecular mass marker (M) was used

reported the accumulation of a 26 kDa protein in the soluble fraction (Fig. 2c), which appeared to be a legumin-type storage protein (identified with the Edman degradation method) that co-migrates with the 26 kDa dehydrin and masks detection of the dehydrin using Western blot analyses (Fig. 2a).

Soluble protein extracts representing subsequent stages of seed development and maturation (Fig. 2c) were analyzed according to the presence of post-translational phosphorylation (Fig. 2d). None of the three detected dehydrins (Fig. 2a) appeared to be heavily phosphorylated during seed development. Proteins with approximate

molecular weights of 23 and 26 kDa seem to be the most abundant phosphoproteins. These phosphoproteins are present during all developmental stages at relatively unchanged levels, which exclude the possibility that the 26 kDa phosphoprotein is the dehydrin-like protein. The possibility of protein phosphorylation was examined for all detected dehydrin-like proteins, and all of them appear to be phosphorylated (Fig. 2e) because their molecular weight decreased after dephosphorylation. A representative dephosphorylation experiment is shown because during all the seed maturation period investigated and in dry seeds similar patterns of protein bands were reported.



◀ **Fig. 3** Dehydrin-like protein localization in the embryonic axes of common beech (*Fagus sylvatica* L.) seeds during development between the 12th and 19th week after flowering. Representative images of subcellular localization from different cell compartments are presented. Using antibody specific to K-segment, dehydrins were localized along the plasma membrane (a), inside cytoplasmic vesicles or small vacuoles (b, c), associated with the amyloplast matrix, envelope, and starch grains (d, e), and membranous structures (f), inside the nucleus (d, g), attached to the mitochondrial envelope (h), throughout the cytoplasm (i), and inside larger, possibly developing storage vacuoles (j). Dehydrin-like proteins are detected with gold particles indicated by arrows. V vacuole, Am amyloplast, N nucleus, M mitochondria, Cw cell wall, Lb lipid bodies. To be consistent with the developmental embryo stage from particular images description: 13WAF (A1, B, D, H); 15WAF (C,E,F); 16WAF (A2, G); 17.5WAF (I, J)

Immunolocalization of Dehydrin-like Proteins

Immunolocalization of dehydrin-like proteins was performed on the embryonic axes of common beech (*F. sylvatica* L.) seeds. Subcellular localizations were analyzed in maturing seeds and dry-stored seeds with antibody specific to dehydrin K-segment. There were no specific dehydrin localizations associated with specific beech seed developmental stages, and there were no differences at the subcellular level in all analyzed tissues from root-hypocotyl axis; however, for this work we focused on the cortical region. All of the examined subcellular localizations were positive for dehydrins throughout all of the seed maturation time points beginning at the 12th up to the 19th WAF. Dehydrin-like proteins were frequently detected along the plasma membrane in the close vicinity to the cell wall (Fig. 3a). In many cells, dehydrin-like proteins were observed as associated with small cytoplasmic structures and attached to vesicles located near cell plasma membrane or were frequently noticed inside those vesicles or in the small vacuoles (Fig. 3a, b, c, f). Moreover, dehydrins appeared to be associated with amyloplasts, being detectable in the developing starch grains, plastid matrix and a lesser extent attached to envelopes (Fig. 3d, e). Amyloplasts were mostly found during seed maturation, and consisted of few smaller size starch grains, especially closer to the radical tip. In general, dehydrins were linked to various membrane structures (Fig. 3f). Specific signals inside the nucleus and their association with scattered chromatin (Fig. 3g) pointed to nuclear localization of dehydrin-like proteins. Dehydrin-like proteins were attached to the mitochondrial envelope (Fig. 3h) and were identified throughout the cytoplasm, especially in the electron dense areas and in the vicinity of lipid bodies and vesicles, located along or close to cell walls (Fig. 3i) and in the larger vacuoles, which are supposed to be developing protein storage vacuoles (Fig. 3j).

Subcellular localization was also investigated in the embryonic axes of stored beech seeds. For this experiment,

2-years-stored seeds were used, and at this time, fewer specific localizations were found in the dry seed tissues. Dehydrin-like proteins were found mostly inside the amyloplasts, containing from one to three large starch grains per section (Fig. 4a, c). At a greater distance from the radical tip, cortex cells from hypocotyls contained large, mostly simple amyloplasts that also showed a dehydrin positive reaction. Slight dehydrin localization was noticed in the nuclei and cytoplasmic vesicles (Fig. 4b). The dehydrin localization was occasionally detected inside the vacuoles with dispersed electron dense content, cytoplasm or the plasma membrane, and membranous structures as single gold particles (Fig. 4b, d).

Before the determination of the subcellular locations of dehydrins, to clearly recognize the analyzed structures, we used TEM images after osmium tetroxide treatment and after embedding the material in Spurr's resin (Fig. 5a–d). The material was analyzed from the radical tips to the hypocotyls. The results of the control reaction to assess the specificity of labeling were negative (Fig. 5e–h); we did not observe any gold particles in the structure analyzed.

Prediction of Possible Dehydrin Interactions

Dehydrin/response ABA protein from *F. sylvatica* with accession number CAE54590.1 (Jiménez and others 2008) and dehydrin, partial from *F. sylvatica* with accession number CBY89194.1 (unpublished data) are the only accessible dehydrin sequences in public databases from beech seeds. Using the full dehydrin/response ABA protein sequence as a query, the ability of dehydrin protein to interact with DNA was investigated with the DIsis program. The RKKK residues at position 124–127 were indicated as the most probable sites of interaction. The DP-BIND program indicated 5 amino acid tracks, including the RKKK sequence, and 12 single amino acids (Supplementary Table 1) with the highest probability of interaction with DNA. The Profisis program was used to predict 12 potential sites of dehydrin-protein interactions (Supplementary Fig. 1), and 5 of them were represented by tracks of 4–16 amino acids. Based on the amino acid sequence, dehydrin/response ABA protein (CAE54590.1) has a 12.5 % glycine content, which indicates it is a highly hydrophilic protein.

Monte Carlo simulations of helical peptides in association with lipid membranes were performed using the MCPep server to predict putative dehydrin-lipid interactions (Supplementary Fig. 2). Three 50 amino acid dehydrin (CAE54590.1) peptides were analyzed, and two of them contained the consensus K-segment, which potentially forms an amphipathic helix, and one N'-terminal peptide containing no consensus dehydrin segments as a reference probe. Two peptides, each containing the

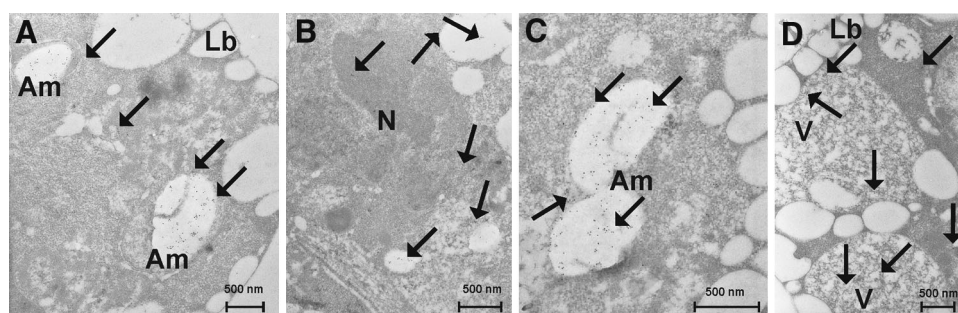


Fig. 4 Dehydrin-like protein localization in the embryonic axes of beech seeds stored for 2 years. Using antibody specific to K-segment, dehydrins were identified (*arrows*) inside the amyloplasts (**a, c**), in the

nuclei (**b**), and associated with vesicles and vacuoles with dispersed electron dense content (**b, d**). *Am* amyloplast, *N* nucleus, *Lb* lipid bodies

consensus K-segment, were predicted to interact with membranes based on the average distance of each residue from the membrane midplane and the capacity to stay in a water solution (Supplementary Fig. 2a–d). Simulations of the N'-terminal peptide of dehydrin showed that it does not interact with membranes but stays in water solutions (Supplementary Fig. 2e, f). Moreover, it was predicted that the peptide containing the K-segment in position 125–140 may be closer in proximity (20 Å) to the phosphate groups of the lipid polar heads than the peptide containing the K-segment in positions 168–182 (40 Å) (Supplementary Fig. 2g).

Discussion

Accumulation of the 44 kDa dehydrin was previously assumed to be an important embryogenesis program element associated with the acquisition of desiccation tolerance during beech seed development (Kalemba and others 2009). This protein was the most abundant among all detected dehydrin-like proteins (Fig. 2a). At 19 WAF, the concentration of the 44 kDa dehydrin increased over 15 times compared to the initial level (12–13 WAF), and the increase was strongly negatively correlated with the water content of embryonic axes (Fig. 1). Dehydrins are a multi-family of plant proteins related to embryogenesis, and they accumulate under water stress conditions (Rorat 2006; Battaglia and others 2008; Hara 2010; Battaglia and Covarrubias 2013). Preferential hydration of diverse molecules and water replacement in dehydrated cells is performed by dehydrins to maintain structural stabilization (Hoekstra and others 2001), including during the development and maturation of seeds (Panza and others 2007).

Nuclear localization, particularly in the segmented nuclei, irregular in shape and characteristic of maturing cells, was reported in the embryonic axes of developing beech seeds. This finding indicated the importance of dehydrin-like proteins in the protection of nuclear processes

because they were associated with scattered chromatin. Dehydrins were previously found to be associated with chromatin in the nucleus of *A. angustifolia* (Bert.) O. Kuntze embryos (Farias-Soares and others 2013) and mature *Euterpe edulis* Martius seeds (Panza and others 2007). The dehydrins possibly participated in protection of the transcriptional machinery enabling the realization of the seed developmental program (Panza and others 2007; Farias-Soares and others 2013) by induction and maintenance of embryogenesis (Burrieza and others 2012). Dehydrin–DNA interaction may stabilize the DNA conformation in the nucleus during seed maturation under extreme dehydration (Lin and others 2012), specifically when strongly labeled areas contain dispersed chromatin (Houde and others 1995). Contrary to developing seeds in the embryonic axes of dry, stored beech seeds, we observed only slight dehydrin localization in the nuclei (Fig. 4b). This appears to be common as little dehydrin labeling was detected in nuclear areas containing dense chromatin (Houde and others 1995; Carjuzaa and others 2008).

The Y-segment was thought to be a putative nucleotide-binding domain (Close 1996). Using accessible beech dehydrin sequences, we predicted that at least one dehydrin has the ability to interact with DNA through the RKKK residues, which is the motif that resembles the SV40-like NLS sequence (K-R/K-X-K/R), and several other single or neighboring amino acids including a part of Y-segment sequences (Supplementary Table 1). DNA-binding activity of the RKKK residues was proposed by the DIsis and DP-Bind programs and was demonstrated experimentally by Yen and others (1998), which makes it highly probable that there is a DNA-binding site in dehydrin proteins. Yen and others (1998) reported that the RKKK sequence was critical for high-affinity DNA association. Dehydrins, which lack the nuclear localization signal, were also found in the nucleus (Houde and others 1995; Wisniewski and others 1999) indicating that nuclear localization is not always the final destination of dehydrin proteins. Co-transport to the nucleus through protein–protein interaction may explain

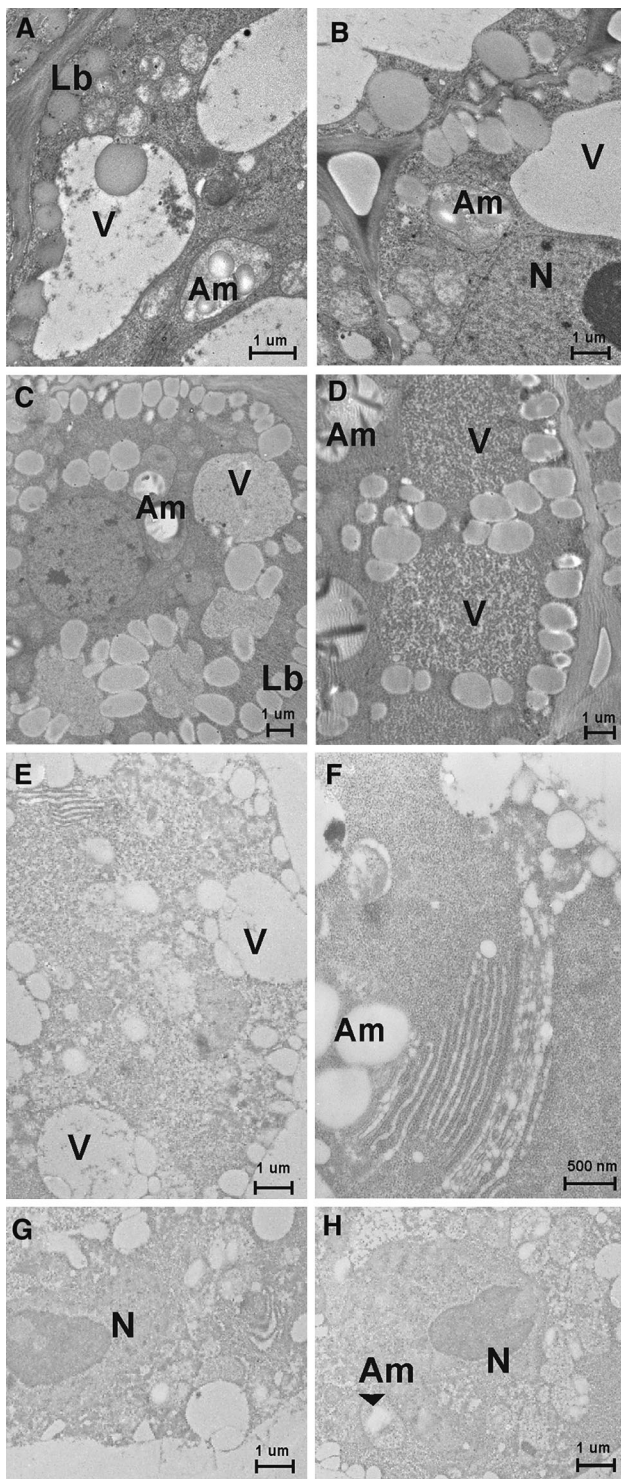


Fig. 5 Ultrastructure of the embryonic axes of beech seeds (material after osmium tetroxide post-fixation and Spurr's resin embedding, **a–d**) and negative results of control reactions (**e–h**). *Am* amyloplast, *N* nucleus, *V* vacuole, *Lb* lipid bodies

the nuclear localization of dehydrins (Houde and others 1995). Conversely, nucleus-targeted proteins, including dehydrins containing the RKKK motif, may function as

nuclear import/export carriers (Godoy and others 1994). This function of dehydrin-protein interactions may be made possible through the predicted amino acid tracks (Supplementary Fig. 1) given the probability of interactions with various types of protein domains and motifs even when dehydrin-protein interactions are labile (Danyluk and others 1998; Heyen and others 2002). Jensen and others (1998) demonstrated that phosphorylation of the Rab17 dehydrin protein by CK2 kinase is an essential step for its nuclear location. Moreover, Rab17 was the most heavily phosphorylated protein in the mature maize embryo (Godoy and others 1994). Our results showed that two phosphoproteins dominate during beech seed development (Fig. 2d). Although dehydrins were not the most highly phosphorylated proteins, it is likely that all detected dehydrin-like proteins (Fig. 2a) contained polyserine segments and were present in seeds in their phosphorylated forms (Fig. 2e), which is the major post-translational modification and is required for protein function (Brini and others 2007). Dehydrin protein (CAE54590.1) was classified in the Y_nSK_2 group (Jiménez and others 2008), and its molecular mass predicted from the amino acid sequence is similar to the molecular weight of the 26 kDa dehydrin after enzymatic dephosphorylation (Fig. 2e). The band pattern after dephosphorylation indicates that this protein may comprise several phosphorylation states (Jensen and others 1998) related to numerous serine residues in the sequence (Jiménez and others 2008).

It has been demonstrated that water removal initiates contacts between dehydrins and membrane lipids (Danyluk and others 1998). Cell membranes are regarded as the main site of desiccation injury because disorganization of membranes apparently results in enhanced permeability. In maturing beech seeds, electrolyte leakage was 4–6 times higher at 12 and 14 WAF, which is before the acquisition of desiccation tolerance (Kalemba and others 2009). At this time, a high water content (Fig. 1) and only the 37 kDa dehydrin-like protein were detected (Fig. 2). At the 16th WAF, increased 26 and 44 kDa dehydrin levels (Fig. 2a, b) were linked with lowered electrolyte leakage (Kalemba and others 2009). This observation may be linked to the plasma membrane localization of dehydrin-like proteins after protein accumulation (Fig. 2a). For dehydrin–membrane interactions, it is unknown whether membrane lipids or membrane proteins are the main target. However, beech dehydrin protein was predicted to interact with other proteins (Supplementary Fig. 1) and lipid membranes (Supplementary Fig. 2). Dehydrins are peripheral proteins (Danyluk and others 1998) and can function as dimers (Lin and others 2012). The lack of certain tertiary structures in dehydrins additionally favors the potential of such interactions (Kovacs and others 2008). Koag and others (2003) suggested that dehydrin function may be related to their

membrane targets as nearly all of the structure gained by dehydrins upon binding to small uni-lamellar vesicles (containing acidic phospholipids) were α -helical. Dehydrin association with the cell membrane may be explained by the fact that dehydrins can favorably bind to lipid vesicles of smaller diameters (Koag and others 2003). The K-segment is predicted to interact with membranes by forming an amphipathic α -helix (Close 1996; Koag and others 2009), which is required for binding to anionic phospholipid vesicles (Koag and others 2009). All detected dehydrin-like proteins contained at least one K-segment (Fig. 2a); therefore, their possible association with membranes through K-segments is likely (Supplementary Fig. 2). The endomembrane system in plant cells has a complex organization (Ebine and others 2008). The dehydrin-like proteins associated with small vesicles located near the cell membrane and small structures throughout the cytoplasm may participate in cellular transport and membrane fusion, including protein transport into forming protein storage vacuoles (Chrispeels 1985). The protein inclusions inside storage vacuoles were also observed in this work (Fig. 5a), thus possibly some of the vesicles with dehydrins detected are suspected to be transported into storage vacuoles. Accumulation of dehydrin proteins near membranes and/or cell walls during osmotic stress is a well-known phenomenon not only in plants but also in the moss *Physcomitrella patens* (Ruibal and others 2012) and the fern *Polypodium polypodioides* (Layton and others 2010). Accumulation of WCOR410 proteins in the vicinity of the plasma membrane of cold-acclimated wheat cultivars suggested that they were involved in the cryoprotection of the plasma membrane against freezing or dehydration stress (Danyluk and others 1998). In contrast, Borovskii and others (2005) established that during autumnal hardening or adaptation to low temperature, dehydrins were bound to the outer mitochondrial membrane in wheat crowns. In our studies, dehydrins were identified as associated with the mitochondrial envelope, possibly both on the inner and outer sides, in a manner unrelated to low temperature. For instance, constitutive accumulation of putative dehydrins in the mitochondrial matrix of lupin seeds was possibly related to the protection of important soluble mitochondrial enzymes (Rurek 2010). It would be interesting if dehydrins could neutralize cellular oxidative stress acting at the border of mitochondria, the main reactive oxygen species source, and at the cytoplasm specifically when the mitochondria are intensively producing energy during seed development. This antioxidant hypothesis of some types of dehydrins is supported by the results showing that dehydrin protein prevented peroxidation of liposomes in vitro (Hara and others 2003) and disrupted DrpA (dehydrin-like gene) mutants of *Aspergillus fumigatus* that were hypersensitive to oxidative stress

(Wong Sak Hoi and others 2011). Therefore, dehydrin-like proteins may act as radical-scavenging molecules (Hara and others 2003) that protect mitochondrial membranes under stress and may be involved in the control of the physiochemical status of mitochondrial membranes (Rurek 2010).

The unique amyloplast localization of dehydrins in the embryonic axes of maturing and stronger accumulation in dry-stored beech seeds exemplifies the fact that dehydrin subtypes can accomplish their function in different cell compartments (Danyluk and others 1998; Vornam and others 2011). In non-cold-acclimated apices of birch, dehydrins were present only in the cytoplasm, whereas in cold-acclimated plants, dehydrins were detected in nuclei and storage protein bodies (Rinne and others 1999). In birch, dehydrin production in the apices is accompanied by starch grain production, and dehydrins were strongly accumulated in starch-rich amyloplasts (Rinne and others 1999). Similarly, in cold-acclimated wheat plants, dehydrins were located preferentially at the plasma membrane lining, and at the same time, no labeling was detected in the cytoplasm, nucleus, organelles, or vacuoles (Danyluk and others 1998) confirming that one single dehydrin location may occur under specific conditions. Dry embryonic axes of beech seeds contained no more than 10 % water and thus consist of desiccated tissue. Recently, it was shown that dehydrins play a role in desiccation tolerance (Wang and others 2012), and this function is conserved in stored beech seeds (Kalemba and Pukacka 2014). Dehydrins associated with amyloplasts may act by rescuing enzyme functions in key metabolic processes during dehydration. These enzyme functions are required for survival and re-growth by creating a local pool of water in dehydrated cells (Rinne and others 1999; Jiang and others 2001). The rescue of enzyme functions by dehydrins during dormancy alleviation and germination of beech seeds enables starch utilization enzymes to function, which creates an early energy source. Dehydrins associated with amyloplasts (Fig. 4a, c), protein storage vacuoles (Rinne and others 1999), and lipid bodies (Egerton-Warburton and others 1997) emphasize the significant roles of dehydrin in storage reserve accumulation and subsequent consumption. The buffering of digestive enzymes early in germination by dehydrins is important, as protein bodies were noted to be directly involved in storage lipid mobilization during seed germination by shifting lytic enzymes from protein bodies to the cytoplasm and the surface of the oil bodies after seed imbibition (Zienkiewicz and others 2014).

At some stage of desiccation after the loss of structural water, important processes such as water replacement and glassy structure formation are initiated (Hoekstra and others 2001). LEA proteins participate in all of these processes and lead to water deficit tolerance expansion by acting as a hydration buffer (Sivamani and others 2000). Additionally,

LEA proteins increase the glass transition temperature and the strength of hydrogen bonds of the glass matrix (Wolters and others 2001). It was shown that after desiccation in the embryonic axes of beech seeds, the glassy state is formed in the cytoplasm (Pukacka and others 2003). However, the cytoplasmic localization of dehydrins in the dry state was not reported. This is curious because at least one beech dehydrin (CAE54590.1) is highly hydrophilic because a protein can be classified as hydrophilic, when the glycine content is above 6 % (Battaglia and others 2008). However, this does not exclude the possible action of dehydrin-like proteins in glassy state formation even if they are not present in the matrix structure.

Egerton-Warburton and others (1997), investigating embryo tissue of *Zea mays* L. kernels, stated that the primary function of dehydrins involves the cytosol and the structures contained therein. After analyzing all published data presenting the different subcellular immunolocalizations of dehydrins, it can be hypothesized that dehydrins are easily transported throughout the cell, and their destination is variable and depends on the cell water state, stage of development, and many genetic and environmental factors. Afterward, dehydrins are recruited to ameliorate different stress effects (Rorat 2006; Battaglia and others 2008; Hara 2010; Battaglia and Covarrubias 2013) including plant–pathogen interaction (Zhai and others 2011) as rice dehydrin (specifically K-segment) exhibited antibacterial activities against Gram-positive bacteria. The highest density of gold particles was found inside vacuoles. VCaB45, a phosphorylated 45 kDa calcium-binding dehydrin containing the K-segment, was located within the lumen of vacuoles (Heyen and others 2002). It was proposed that VcaB45 plays a calcium-dependent protein chaperone role similar to the function of calnexin and calreticulin. In our studies, ion binding activity was not analyzed. However, dehydrin-like proteins may act as chaperones to molecules deposited in the vacuole during the maturation of beech seeds.

Conclusion

It has been established that LEA proteins accumulate in high concentrations in embryo tissues during the last stages of seed development before desiccation, and specifically, dehydrins are regulators of cellular osmotic potential. In our research, dehydrin-like proteins have been localized in various cell parts and organelles during seed developmental stages and when seeds were desiccated and stored. These findings highlight that particular molecules, organelles, or biological processes have to be protected during different seed developmental stages. Dehydrin-like proteins appear to be involved in the reduction of desiccation-

induced cellular damage of the plasma membrane, organelle membranes, nucleus, and storage material reservoirs. Dehydrins most likely also assist and are a part of vesicle-mediated cellular transport during seed development and assist in storage material degradation early in germination, which emphasizes their ability to associate with lipids and cell membranes, consisted of the phospholipid bilayer with embedded proteins. However, dehydrin–membrane interactions are of particular interest for future investigation because, apart from plasma membrane and membrane structure localizations, dehydrin-like proteins were associated with organelle membranes and found within organelles, and they displayed a linear pattern. Potential membrane-associated dehydrin-like protein partner interactors, predicted with *in silico* analyses, are still unknown. This is a notable research area requiring future investigation to elucidate the mechanism of dehydrin chaperone activity.

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Conflict of interest The authors declare that they have no conflict of interest.

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