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Association of Protective Proteins with Dehydration and Desiccation of *Orthodox* and *Recalcitrant* Category Seeds of Three *Acer* Genus Species

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Abstract Mature and dried seeds from three species of the *Acer* genus, which differed in desiccation tolerance, were analyzed. The three species investigated were as follows: *Acer platanoides* L. (Norway maple, *orthodox*, A1 and A2 seedlots); *Acer pseudoplatanus* L. (sycamore, *recalcitrant*, B1 and B2 seedlots); and *Acer saccharinum* (silver maple, *recalcitrant*, C1 and C2 seedlots). We compared the appearance of dehydrins and small heat shock proteins in seedlots originating from cropping years that differed in weather conditions, which were monitored in detail during seed development. The experiments showed that three main dehydrins with approximate molecular weights of 46, 35, and 23 kDa were characteristic of all examined *Acer* species seeds. The three proteins were present in the A1 and A2 seedlots of the *orthodox* category Norway maple seeds and were noted either individually or together in the B1, B2, C1, and C2 seedlots of *recalcitrant* category seeds. It was found that one major small heat shock protein existed with a molecular mass of 22 kDa and was detectable at high concentrations in all seeds of the studied *Acer* species; after the seeds were dried, the content of this protein significantly increased. The potential modulation of dehydrin expression by environmental factors such as developmental heat sum and rainfall is discussed in the present work. The influence of water removal, which is caused by seed drying, in seeds of the same genus and belonging to the *orthodox* and *recalcitrant* categories is also explored.

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Introduction

Seeds of various plant species differ in water stress tolerance. *Orthodox* seeds acquire desiccation tolerance (DT) during development and may be stored in the dry state for predictable periods under defined conditions (Roberts 1973). *Recalcitrant* seeds are only drought-tolerant and remain desiccation-sensitive during development and after shedding. Recalcitrant seeds cannot resist the effects of drying or low temperatures; thus, they cannot be stored for long periods because they may lose viability (Roberts 1973; Berjak and Pammenter 1994). Some seeds do not display all the features that are reported for both categories and present intermediate seed storage behaviors (Ellis and others 1990).

Important tree species found in the temperate zone include the Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.), and silver maple (*Acer saccharinum* L.). These trees represent the genus *Acer* but differ in seed physiology, specifically, in their tolerance to dehydration. Norway maple seeds become tolerant to desiccation 18 weeks after flowering (Pukacka and Wójkiewicz 2002). Subsequently, these seeds can be slowly desiccated at ambient temperature until they reach a moisture content (MC) of 8–10% without a loss of viability. Norway maple seeds clearly belong to the *orthodox* category, whereas sycamore seeds are classified as *recalcitrant* (Hong and Ellis 1990; Dickie and others 1991). Sycamore seeds remain sensitive to desiccation throughout the development period (Dickie and others 1991), and at the end of maturation, these seeds maintain a high MC of 50-55% (Pukacka 1998).

Sycamore seed viability can be retained after seed-drying up to a 15% MC (Jahnel 1955); however, dehydration below a 27% MC leads to a significant decrease in seed germinability (Pukacka and Czubak 1998). Daws and others (2006) found that the DT of sycamore seeds depended on the heat sum during seed development; they found that the higher the value of the heat sum, the more tolerant the sycamore seeds were to desiccation. Silver maple seeds are also classified as *recalcitrant* and, in contrast to the sycamore and Norway maple, are nondormant. *Recalcitrant* seeds of different or individual species present a wide range of variability in sensitivity to desiccation (Berjak and Pammenter 1994). For example, silver maple seeds are more sensitive to desiccation than sycamore seeds (Pukacka and Ratajczak 2006). Recently, some experiments showed that seed recalcitrance could be improved. DT can be induced in normally *recalcitrant* silver maple seeds by treating the seeds with abscisic acid (ABA) and tetcyclasis, which lead to the improvement of seed germinability and dehydrin synthesis (Beardmore and Whitle 2005). Additionally, an experiment that included the treatment of silver maple seeds with a solution of selenium ions before drying showed that the germination capability of this seed could be increased (Pukacka and others 2011).

Many transcripts, proteins, and carbohydrates accumulate during seed drying and desiccation, including late-embryogenesis abundant proteins (LEA) and, specifically, dehydrins (group 2 of the LEA proteins) (Close 1996). The preferential hydration of membranes and macromolecules is the primary mechanism acting in drought tolerance, and it involves many protective molecules. Important processes such as water replacement and glassy structure formation are initiated at a specific stage of desiccation after the loss of structural water (Hoekstra and others 2001). LEA proteins participate in all of these processes and work to expand the tolerance of water deficit by acting as a hydration buffer (Sivamani and others 2000). Dehydrins are characterized by highly conserved sequences, including a lysine-rich K segment, a polyserine S segment, a Y segment, and a Φ segment. The structural and functional characteristics of LEA proteins have been identified to understand their role during the adaptive response to water deficit in plants (Rorat 2006; Battaglia and others 2008). The K segment uniquely contains the consensus amino acid sequence EKKGIMDKIKELPG, and it is present in all types of dehydrins (Close 1997). Despite dehydrins' conserved sequences, they appear to be intrinsically disordered proteins that may exert chaperone activity through an "entropy transfer" mechanism. Structural disorder confers advantages to proteins, such as an increased speed of interaction, the combination of specificity with weak and reversible binding, and the ability to carry out more than one function (Kovacs and others 2008). The proteins may have roles as antioxidants and membrane and protein stabilizers during water stress, either by direct interaction or by acting as molecular shields (Tunnacliffe and Wise 2007). The phosphorylation of dehydrins is a major post-translational modification that is required for protein function (Brini and others 2007). Dehydrins are synthesized during seed development as an element of the embryogenesis program, and their accumulation is related to the acquisition of DT (Close and others 1993; Kalemba and others 2009). Furthermore, dehydrin expression can be induced in seeds by drought and ABA (Nylander and others 2001). Dehydrins and dehydrin-related proteins were detected in many plant seeds, including the *orthodox* woody plant seeds of *Acer platanoides* L. (Finch-Savage and others 1994; Greggains and others 2000); intermediate *Fagus sylvatica* L. seeds (Kalemba and Pukacka 2008); and many *recalcitrant* woody plant seeds of *Castanospermum australe* L., *Clausena lansium* (Lour.), *Castanea sativa* L., *Aesculus hippocastanum* L., *Acer pseudoplatanus* L., *Acer saccharinum* L. (Finch-Savage and others 1994), *Quercus robur* L. (Finch-Savage and others 1994; Sunderlíková and others 2009), *Euterpe edulis* (Panza and others 2007), and *Quercus petraea* (Matt.) Liebl. (Vornam and others 2011). Interestingly, Finch-Savage and others (1994) reported that *recalcitrant* seeds from the *Acer* genus contained more dehydrins than *orthodox* seeds. Most dehydrin-like protein bands were detected in silver maple seeds; however, Greggains and others (2000) found that only one dehydrin protein with a molecular weight of approximately 20 kDa was present in Norway maple, which contained two additional dehydrin proteins, and in sycamore seeds.

Resistance to desiccation is possible in the presence of LEA proteins, small heat shock proteins (sHSPs) (DeRocher and Vierling 1994), specific mono-, di-, and oligosaccharides, and soluble sugars (Hoekstra and others 2001). Mechanisms related to ROS removal (Bailly 1994) and glass formation (Buitink and others 1998) also contribute to this resistance. sHSPs are ubiquitous stress proteins that are proposed to be chaperones and, in plants, are encoded by an unusually complex gene family (Siddique and others 2008). This protein family is localized to the cytosol, nucleus, plastids, endoplasmic reticulum, and mitochondria (Vierling 1997; Sun and others 2001). sHSPs are synthesized during embryogenesis, germination, pollen production, and fruit maturation (Sun and others 2001). Additionally, these proteins can be associated with the general mechanism of cellular response to abiotic stress and heat shock because their expression can be induced by oxidative stress, cold stress, heavy metals, ozone, UV, and radiation (Waters and others 1996; Sun and others 2001; Kalemba and Pukacka 2007). sHSPs can attach to denatured proteins, prevent aggregation (Waters and others 1996; Halsbeck and others 2005), stabilize conformations,

and assist in protein folding, oligomer formation, and intracellular transport (Hendrick and Hartl 1995), all of which are important during drying and desiccation.

In the present study, an attempt was made to determine if the appearance or expression of protective proteins such as dehydrins or sHSPs is involved in defining physiological differences between *orthodox* and *recalcitrant* seeds of *Acer* species. Seeds of three *Acer* species that were harvested during various cropping years were compared and analyzed to determine if a genetic or an environmental influence dominated the regulation of protein expression in these organisms.

Materials and Methods

Plant Material

The seeds were collected from single Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.), and silver maple (*Acer saccharinum* L.) trees in the Kórnik Arboretum (Poland) during the growing seasons from 2004 to 2008. Seedlots originating from different species and growing seasons were marked with a capital letter and subsequent number (Table 1). Weather conditions such as the heat sum and rainfall are given in Table 2. The heat sum was calculated from the average temperatures for months, when intense development of seeds occurred in all tested *Acer* species. Duration of the time of embryogenesis and maturation of sycamore seeds was calculated according to the observation of seed embryo formation (Pukacka 1998). Temperature and rainfall measurements were obtained from the Institute of Meteorology and Water Management (IMGW), Poznań Department, Kórnik Station. Seeds were collected immediately after they matured. After harvest, seeds were transported immediately to the laboratory in sealed plastic bags. A portion of the seeds (intended for germination tests and protein detection after desiccation) were subjected to dehydration, and the rest were immediately extracted from the pericarp and seed coats. The moisture content and dry mass were determined using three samples of 10 cotyledons and 20 embryonic axes that were dried at 100°C for 24 h. Suitable samples of embryonic axes and cotyledons for the determination of certain proteins were weighed and stored at −80°C until use.

Table 1 Seeds collected from Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.), and silver maple (*Acer saccharinum* L.) trees during the growing seasons from 2004 to 2008 were assigned as seedlots marked with a capital letter and subsequent number

Species	2004	2005	2008
Norway maple	A1		A2
Sycamore	B1		B2
Silver maple	C1	C2	

Germination Test

A germination test was performed on four samples of 50 seeds each. Freshly collected and dried seeds were hydrated at 100% relative humidity (RH) for 24 h. Fully imbibed seeds were placed between moist rolled paper towels in separate boxes and incubated at 3°C. Stratification was conducted for 18 weeks. A seed was scored as germinated when the radicle protruded 5 mm. Germination counts were made every week, and germinated seeds were removed (ISTA 1999).

Protein Extraction

Embryonic axes and cotyledons were separated and 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 7 µl of protease inhibitor cocktail (Sigma-Aldrich, Poland, P-9599) for each 0.2 g of plant tissue and polyvinylopolypyrrolidone (Sigma-Aldrich, Poland); the samples were further centrifuged (20,000 *g* at 4°C for 20 min). To obtain heat-stable proteins, the supernatant was boiled for 10 min, cooled on ice for 15 min, and centrifuged as described above. Protein concentration was measured according to Bradford (1976).

Table 2 Heat sum and rainfall during development of sycamore (*Acer pseudoplatanus*) and silver maple (*Acer saccharinum*) seeds

	Heat sum		Rainfall (mm)	
Sycamore seeds	B1 seedlot	B2 seedlot	B1 seedlot	B2 seedlot
1. Embryogenesis	65.78	71.25	195.3	97.1
2. Maturation	61.19	55.65	154	180.5
Development (1 + 2)	126.97	126.9	349.3	277.6
Silver maple seeds	C1 seedlot	C2 seedlot	C1 seedlot	C2 seedlot
Development	24.57	26.04	78.2	84.5

The quantity of heat-stable proteins was expressed as a percentage of the heat-stable to soluble proteins. Proteins were resolved with 15-17% SDS-PAGE (Laemmli 1970) and stained with Coomassie Brilliant Blue R-250 (Sigma-Aldrich).

Western Blotting

The protein extract (12.5 μg) was calculated based on the calibration curve data (Marian and others 2003) and loaded onto a gel. Fractioned proteins were transferred onto a polyvinylidene fluoride membrane (Immobilon™-P, Millipore) at 350 mA for 1 h and were then blocked and incubated with a primary antibody raised against the dehydrin consensus K segment (Close and others 1993) and *Arabidopsis* Hsp 17.4 (Wehmeyer and others 1996). The secondary antibodies were conjugated with alkaline phosphatase (Sigma-Aldrich) and visualized by reaction with an alkaline phosphate substrate (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium) (Sigma-Aldrich). All primary antibodies were raised in rabbits. Protein molecular mass was estimated according to the Prestained Protein Molecular Weight Marker (Fermentas).

Densitometry Analysis

The densitometry data were prepared using the BIO 1D++ (Vilber Lourmat) program with a Vilber Lourmat apparatus. Densitometry image analysis was based on the digitalization of the image in pixels whose intensity is 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), and presented in 1×10^{-3} units obtained from $V = \Sigma n_i I$, and from the number of pixels inside the area of the spot.

Alkaline Phosphatase Treatment

Alkaline phosphatase treatment was performed on the heat-stable protein fractions. For dephosphorylation, 100 μg of proteins from this fraction was treated with 4 units of calf intestine alkaline phosphatase (CIAP) (Sigma-Aldrich). The reaction was performed in CIAP buffer at 37°C for 16 h.

Statistical Analysis

Data are presented as mean ± standard deviation of three replicates. The statistical differences between particular parameters were tested using a correlation coefficient analysis. The significance amongst the means of the components (between-group component and within-group component) was verified using an F-test at $p < 0.05$.

Results

The analyses of selected proteins with protective or chaperone activities from the seeds of Norway maple (*Acer platanoides* L.), sycamore (*Acer pseudoplatanus* L.), and silver maple (*Acer saccharinum* L.) presented interesting results that were dependent on the seed category. Mature (M) Norway maple (*Acer platanoides* L.) seeds and dehydrated (D) seeds at an 8% moisture content (MC) were compared. The MC of the mature seeds immediately after collection from the tree was 54% (A1 seedlot) or 52% (A2 seedlot). The germination capacity of Norway maple seeds in all analyzed seedlots was 100%, even after drying to an 8% MC (Fig. 1a).

Three heat-stable dehydrins were detected in Norway maple seeds from the A1 and A2 seedlots. Proteins with approximate molecular masses of 46 and 23 kDa were detected in embryonic axes and cotyledons, and the third protein (35 kDa) was present in embryonic axes and was barely detectable in cotyledons (Fig. 2b). Experiments using seeds from the A1 and A2 seedlots showed that the dehydrin content depended on seed MC and increased with seed dehydration (Fig. 2b). This increase was verified using densitometry analyses, whereby the 23-kDa protein displayed the highest content (approximately 50%) (Fig. 2a).

Sycamore (*Acer pseudoplatanus* L.) is an *Acer* species that was tested and produced seeds that were classified into the *recalcitrant* category. Seeds were collected during two crop-years, and the seedlots (B1 and B2) differed in conditions and vitality. Protein analyses were performed for mature seeds, and the seeds were dried to a 24% MC. Dried (24% MC) sycamore seeds from the B1 seedlot (Fig. 1b) had a relatively high germination capacity (86%) in comparison to mature seeds (57% MC) that germinated to 100%; however, this effect was not observed for seeds from the B2 seedlot (Fig. 1b). The initial germination capacity of the mature seeds (50% MC) from this lot was only 68%, and this number decreased to 46% after drying to a 24% MC.

Dehydrins that were detected in sycamore seeds were dependent on the year of harvest. In seeds from the B1 seedlot, one heat-stable 23-kDa protein was detected in embryonic axes after drying (Fig. 3). In mature embryonic axes and in mature and dried cotyledons, this protein was not detectable. After drying, two soluble, LEA-like proteins with molecular masses of 35 and 36 kDa were detected in embryonic axes (data not shown), and the level of the 23-kDa protein increased. When analyzing seeds from the B2 seedlot, three dehydrins were detected in mature and dried seeds, and the molecular masses were identical to proteins that were detected in Norway maple seeds (Fig. 4). The 23-kDa dehydrin yielded the most

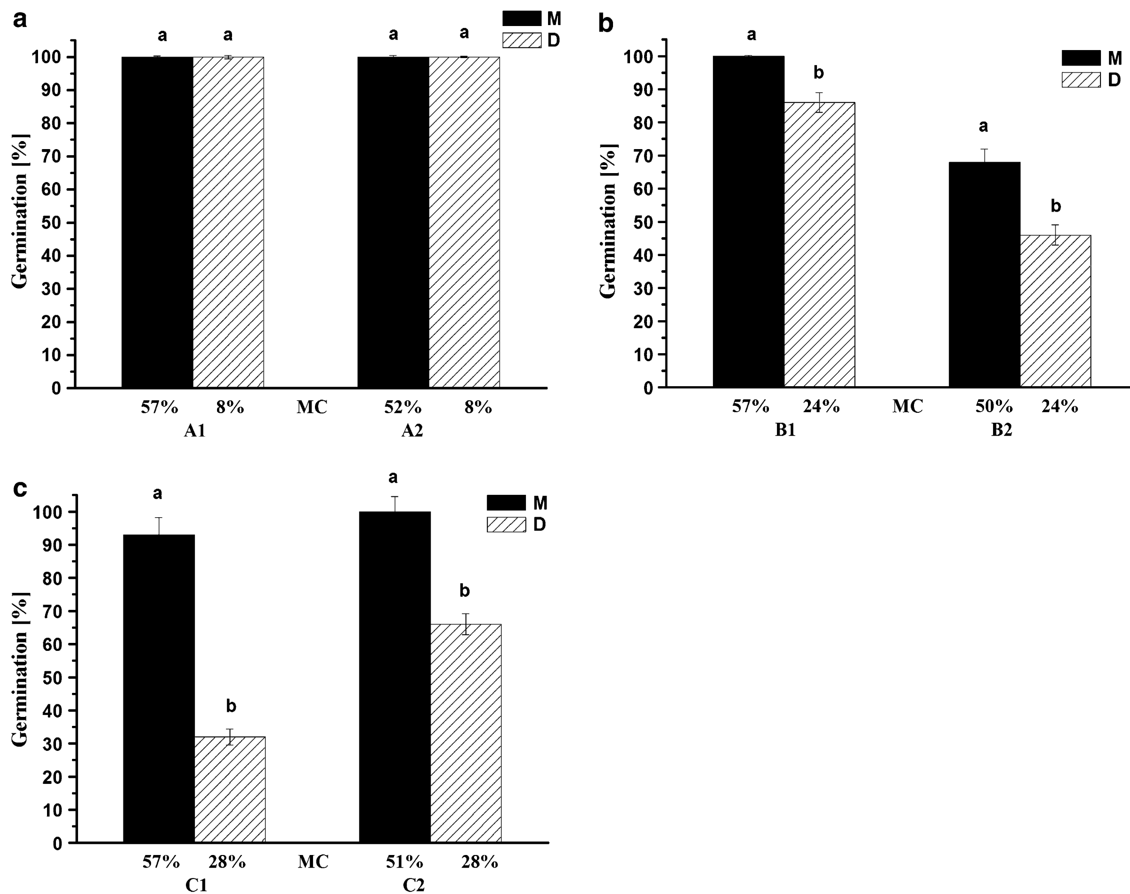

Fig. 1 Germination capacity of mature and dried **a** Norway maple (*Acer platanoides* L.) seeds from the A1 and A2 seedlots, **b** sycamore (*Acer pseudoplatanus* L.) seeds from the B1 and B2 seedlots, and **c** silver maple (*Acer saccharinum* L.) seeds from the C1 and C2 seedlots. Values of the initial MC of the mature and dried seeds are provided in the graphs

intense band after Western blot analysis (Fig. 4a), and the level of detected proteins changed when drying to certain values. Band density analyses confirmed that seeds dried to a 24% MC contained the highest amount of all analyzed proteins (Fig. 4).

Similarly, silver maple (*Acer saccharinum* L.) seeds that originated from the same tree were analyzed during diverse cropping years (C1 and C2 seedlots). After shedding, the collected silver maple seeds from the C1 seedlot were characterized to have 57% MC and a germination capacity of 92%. After dehydration to 28% MC, the germination capacity of the seeds decreased to 32% (Fig. 1c). Dehydrins were present at low levels in silver maple seeds and only in the soluble fraction of the protein extracts from the dried embryonic axes. Two protein bands, with molecular weights of 34 and 35 kDa, were recognized by the dehydrin antibody (Fig. 5a). Seeds of the C2 seedlot were collected and characterized to have a 51% MC and 100% germination capacity. After drying to 28% MC, the ability of these seeds to germinate remained relatively high (66%) (Fig. 1c). In mature, fully hydrated, and dried embryonic axes, three major bands, representing dehydrins with molecular masses of 35, 34, and 22 kDa, were detected (Fig. 5b). In cotyledons, the proteins were detectable at low levels in either the C1 or the C2 seedlot, and dehydration of silver maple seeds from the C2 seedlot did not cause significant changes in the level of detected proteins. Overall, the C2 seedlot contained a higher level of dehydrins than the C1 seedlot (Fig. 5).

All dehydrins that were detected in Norway maple, sycamore, and silver maple seeds were investigated for the occurrence of post-translational modifications. The possibility of protein phosphorylation was examined, and all analyses presented the same banding pattern. The analyses of the protein extracts from Norway maple embryonic axes are presented as a model for all examined seedlots (Fig. 6). The 46-kDa protein was dephosphorylated by enzymatic reaction with phosphatase, and its molecular weight decreased after this reaction. The 35- and 23-kDa dehydrins remained relatively unchanged.

Norway maple seeds contained two main proteins with approximate molecular masses of 36 and 22 kDa, and

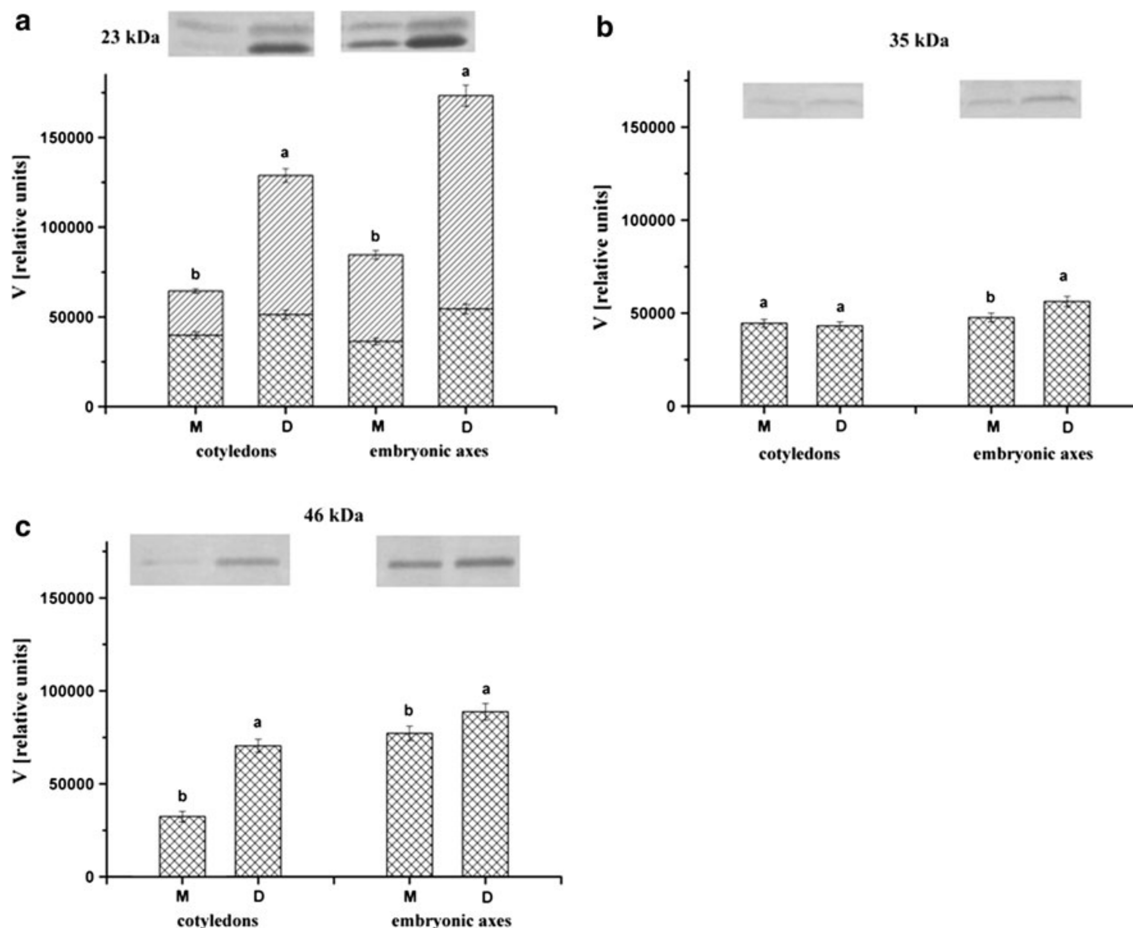

Fig. 2 Dehydrins **a** 23 kDa, **b** 35 kDa, **c** 46 kDa, which were detected in mature (M) and dried (D) Norway maple (*Acer platanoides* L.) seeds from the A1 seedlot. Heat-stable proteins were analyzed, Western blot results are given at the top of each graph, and densitometric analyses of detected dehydrins are presented in graphs. One protein band refers to one respective graph column. Different shading in **a** refers to two protein bands that are estimated to be the 23-kDa dehydrin

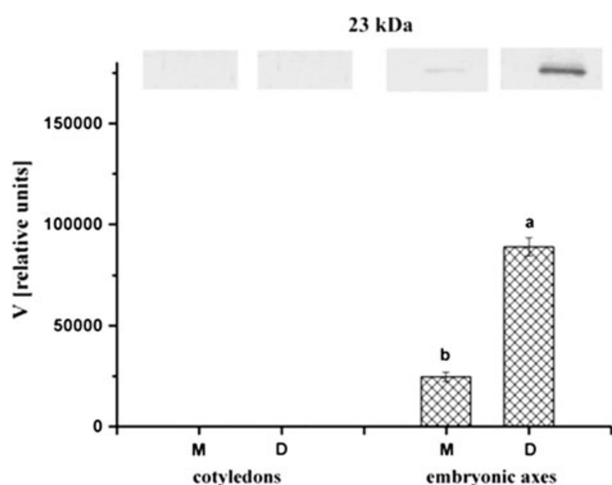

Fig. 3 Dehydrin with an approximate molecular weight of 23 kDa that was detected in embryonic axes of mature (M) and dried (D) sycamore (*Acer pseudoplatanus* L.) seeds from the B1 seedlot

these proteins were recognized by antibodies to small heat shock proteins (sHSP) (Fig. 7a). These proteins were more concentrated in embryonic axes, and after dehydration, their levels increased, and two new polypeptides that were smaller than 20 kDa appeared (data not shown). Changes in sHSP levels were analyzed densitometrically and statistically significant differences were noted. Seed drying caused a considerable increase in sHSP concentration, specifically in embryonic axes where the concentration of these proteins was twofold higher (Fig. 7a). Additionally, sycamore seeds contained one sHSP, similar to the Norway maple. This 22-kDa sHSP was present in cotyledons and embryonic axes (Fig. 7b); however, the protein level was higher in embryonic axes, particularly after drying. Silver maple seeds contained three sHSPs with approximate molecular masses of 36, 24, and 22 kDa (Fig. 7c), and the concentration of the 22-kDa sHSP was the highest of the three. These proteins were detected in

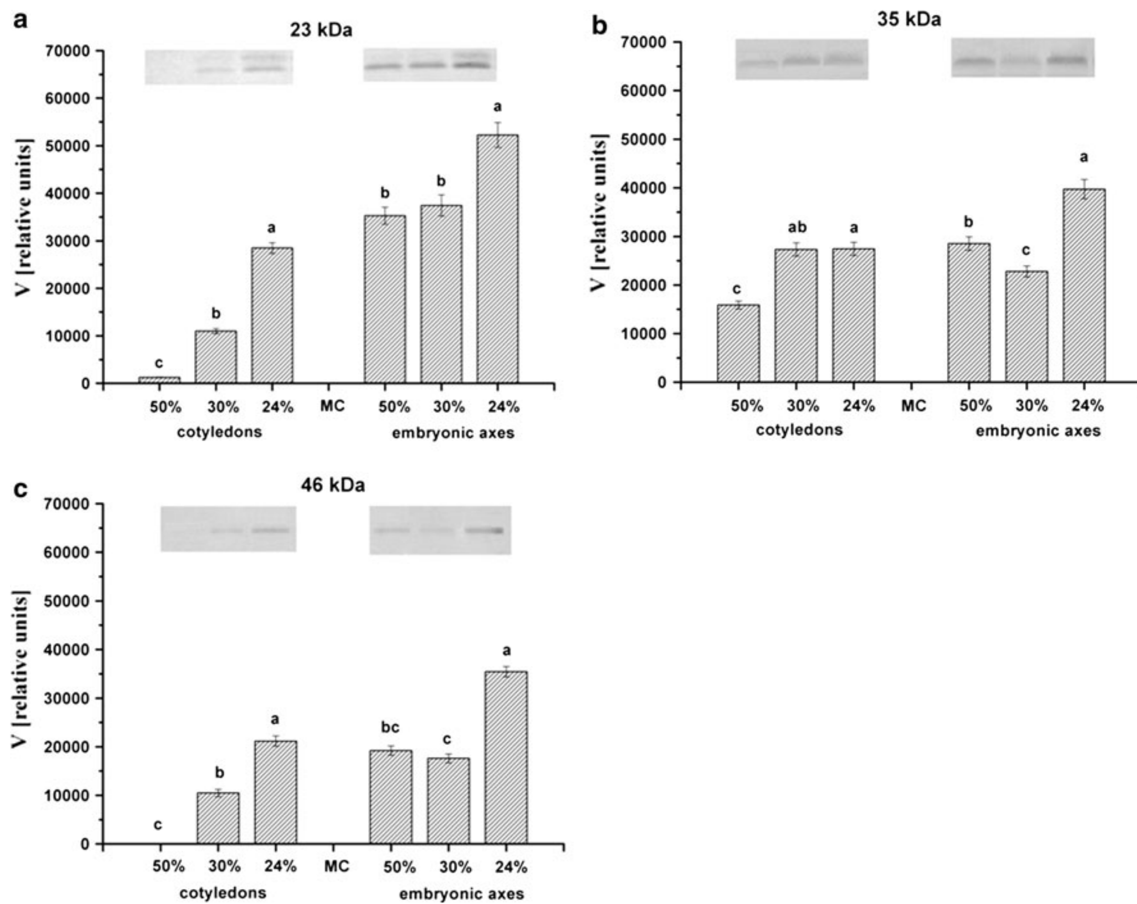

Fig. 4 Densitometric analyses of dehydrins with the molecular weights of **a** 23 kDa, **b** 35 kDa, and **c** 46 kDa, which were detected in sycamore (*Acer pseudoplatanus* L.) seeds from the B2 seedlot. One protein band refers to one respective graph column

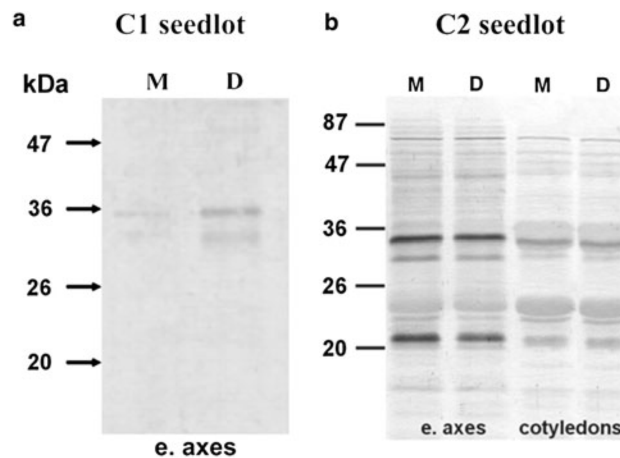

Fig. 5 Dehydrins detected in mature (M) and dried (D) silver maple (*Acer saccharinum* L.) embryonic axes from the C1 seedlot (**a**) and embryonic axes and cotyledons of seeds from the C2 seedlot (**b**)

both cotyledons and embryonic axes of mature and dried seeds, and the concentrations of these proteins were higher in embryonic axes and slightly changed after dehydration (Fig. 7c).

Discussion

Norway maple produces *orthodox* seeds, whereas sycamore and silver maple produce *recalcitrant* seeds. Seeds from

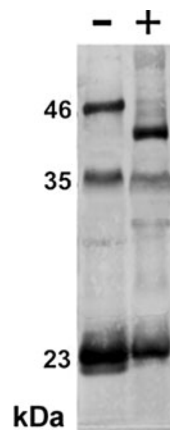

Fig. 6 Detection of post-translational modifications of the 46-kDa dehydrin that were recognized in seeds of three *Acer* species. Protein extracts of embryonic axes of Norway maple seeds before (−) and after (+) enzymatic treatment with phosphatase were loaded onto a gel and analyzed by Western blot to identify occurrences of protein phosphorylation

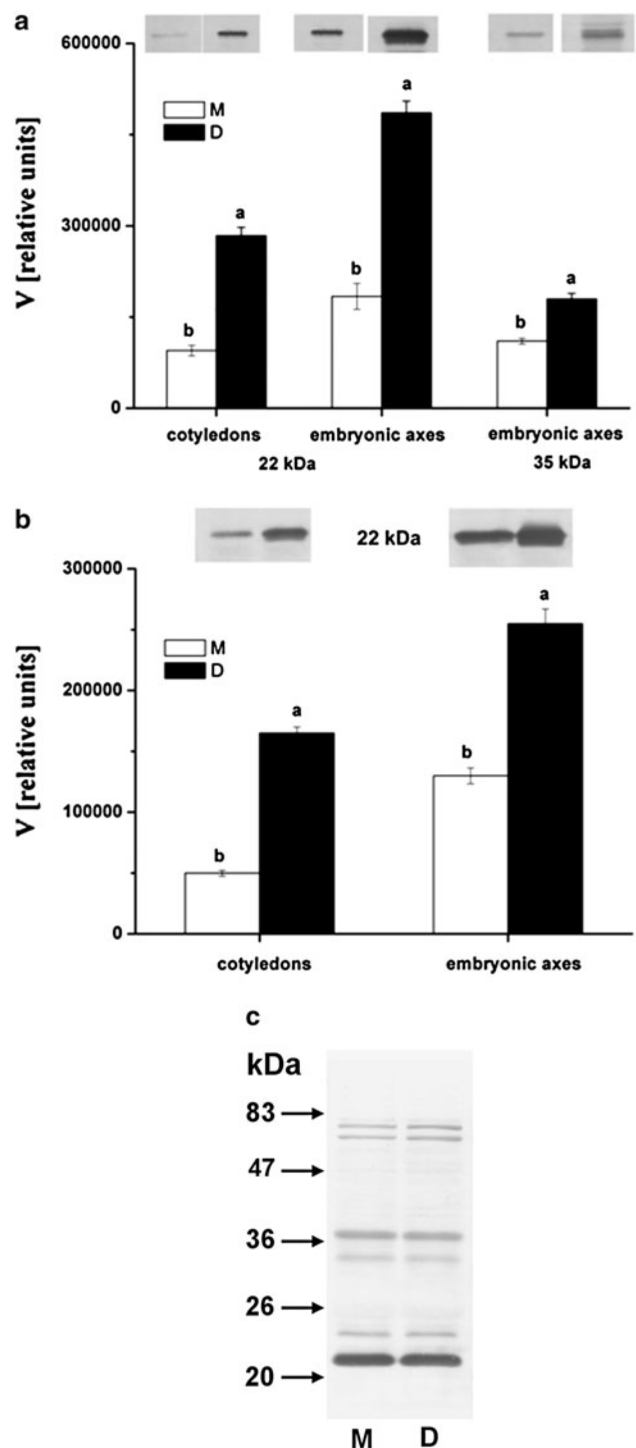

Fig. 7 sHSP proteins, with molecular masses of 22 and 35 kDa, that were detected in **a** Norway maple (*Acer platanoides* L.) seeds; the sHSP protein, with a molecular mass of 22 kDa, that was detected in **b** sycamore (*Acer pseudoplatanus* L.) and **c** silver maple (*Acer saccharinum* L.) seeds. Mature (M) and dried (D) seeds were analyzed

these three species were studied to investigate the similarities or differences in the proteins involved in the cell protection mechanisms that are activated during seed drying. The germination capacity of Norway maple seeds was 100%, even after desiccation to 8% MC (Fig. 1a), whereas the germination capacities of sycamore (Fig. 1b) and silver maple (Fig. 1c) seeds dramatically decreased to below 30% MC after desiccation. Seed drying is described as the reduction of seed moisture content to the suggested levels for seed storage, which will not affect seed viability. Although *recalcitrant* seeds were dried to the recommended level (24-28% MC) (Pukacka and Czubak 1998), the germination capacity of these seeds decreased (Fig. 1b, c). Differences in the germination capacity of the B1 and B2 seedlots after drying may be the result of parental influence (Gutterman 2000). The rainfall was more favorable for the B1 seedlot, specifically during the time of seed embryo formation when the rainfall was twofold higher than at the B2 seedlot (Table 2). Between-year variations in the DT of *recalcitrant* seeds for material collected from the same site have been documented (Finch-Savage and Blake 1994), indicating that environmental conditions can affect a range of seed traits.

Dehydration of plant cells can be environmentally imposed by factors such as drought, cold, or heat shock; additionally, dehydration can be imposed developmentally during seed maturation. Daws and others (2004) analyzed poor DT in *Aesculus hippocastanum* seeds and proposed that one of the external factors that could influence or modify the degree of desiccation sensitivity of these seeds during development was temperature. This group also confirmed the theory that a higher heat sum during *A. pseudoplatanus* seed development resulted in more desiccation-tolerant seeds (Daws and others 2006). Considering that 30% MC was the limit to ensure seed viability, we can theorize that the condition of the B2

sycamore seedlot was low quality because the ability of seeds to germinate after drying was only 46% (Fig. 1b). This result may be the consequence of a low initial germination capacity (68%), which could be caused by poor seed quality, a disease, or an unknown pathogen (not examined). After drying to 28% MC, the silver maple seedlots showed a different germination capacity (C1 was 32% and C2 was 66%) (Fig. 1c). Higher seed viability occurred when the heat sum and rainfall were both higher during the year when the C2 seeds were developing (Table 2). Despite the high amount of rain in this year, there was a long period (21 days) without rain in April, and the total rainfall was only 0.1 mm. Premature drying could also enhance a seed's tolerance to desiccation, and this result was shown in experiments where premature, dehydrated, ATEM6 mutant (group 1 LEA proteins) *Arabidopsis* seeds acquired DT earlier than their wild-type counterparts (Manfre and others 2009). Tolerance to desiccation, as measured by the ability to germinate after desiccation, could be exogenously moderated in seeds. For example, embryonic axes from the normally *recalcitrant* seeds of the silver maple can be made more tolerant to desiccation by pretreatment with ABA and tetcyclasis (Beardmore and Whitle 2005) or selenium (Pukacka and others 2011).

In the present study, the heat-stable fraction of seed proteins was analyzed to detect dehydrins using antibodies that were specific to the K segment (Close and others 1993) and Western blot. The results showed that in the A1 and A2 seedlots of the Norway maple seeds, three dehydrins, with molecular masses of 46, 35, and 23 kDa, could be detected in embryonic axes and cotyledons (Fig. 2). The concentrations of the 46- and 35-kDa dehydrins depended on the seed moisture content and had a general tendency to increase in protein level as desiccation progressed (Fig. 2a, c). The level of the 35-kDa protein remained nearly unchanged in cotyledons but increased slightly in embryonic axes (Fig. 2b), which points to the probable constitutive expression of the protein, as was estimated earlier for the *dhnX* gene and the DHNXERO (YSK_2) mRNA of *Arabidopsis thaliana* (Welin and others 1994). Our analyses confirmed that the 46- and 23-kDa proteins were upregulated by water stress (Fig. 2), and when comparing the A1 and A2 seedlots, the same results were obtained. Additionally, the 23-kDa protein was synthesized in abundance in seeds. In contrast, the 35-kDa protein was found at low concentrations in the cotyledons (Fig. 2b), pointing out that avoiding water stress or repair mechanisms was more successful at regulating these protein levels in embryonic axes than cotyledons. These results are consistent with previous analyses that found that activities of enzymes involved in the ascorbate–glutathione cycle were higher in the embryonic axes than in the cotyledons of desiccated Norway maple and sycamore seeds (Pukacka and Ratajczak 2007).

Dehydrins, a subset of a diverse class of highly abundant, heat-stable LEA proteins, are involved in embryogenesis programming and the stress response, and their role was extensively studied in *orthodox Arabidopsis thaliana* wild-type and mutant seeds (Manfre and others 2009). Seed analyses of desiccation-sensitive tree species appear to be more useful in revealing dehydrin functions. Because dehydrin expression is not limited to only seeds, dehydrins' protective role during cellular dehydration was also examined in various tissues of *Populus alba* × *P. tremula* var. *glandulosa* (Bae and others 2009) and in the petiole, stem, roots, and buds of *Picea glauca* (Richard and others 2000). The *LEA* genes are one possible type of DRE-containing target genes (Almoguera and others 2009). Differential expression of the dehydrin genes is observed in response to various stresses such as drought, cold, salt, and ABA (Rorat 2006; Battaglia and others 2008). The banding pattern of the 23-kDa protein detected in Norway maple seedlots contained two bands that reacted with dehydrin antibodies and had similar molecular weights (Fig. 2a). The proteins are likely the same, and the two bands are not the result of a post-translational protein modification such as phosphorylation (Fig. 6), which can occur in dehydrin proteins at the polyserine segment (Brini and others 2007). However, other types of post-translational modifications or heterozygotic allele expression in the dehydrin gene could explain this banding pattern. Vornam and others (2011) reported allelic variation in the dehydrin gene in natural populations of *Quercus petraea* (Matt.) Liebl. For this reason, it is speculated that two pairs of dehydrin bands (47 and 45 kDa; 22 and 20 kDa), which are detected in silver maple seeds (Finch-Savage and others 1994), might represent two dehydrin proteins.

The following two sycamore seedlots were examined: B1, which was characterized to have a high germination capacity (Fig. 1b) and one 23-kDa dehydrin (Fig. 3), and B2, which was characterized to have a lower ability to germinate (Fig. 1c) and three major dehydrins bands that were similar to the Norway maple (Fig. 4). The accumulation of dehydrins induced by water stress was observed at each sycamore seedlot (Figs. 3, 4), and the differences were most significant for the 23-kDa protein (Fig. 4a). The induction of the synthesis of all dehydrins in the B2 seedlot occurred before maturation drying because those proteins were detected in seeds that had 50% MC (Fig. 4). The highest amount of dehydrin proteins in sycamore seeds were observed in seeds that were desiccated to 24% MC (Fig. 4c), and these increased levels could be the result of the high stability of the dehydrins and/or degradation of proteins that are more sensitive to water removal rather than de novo synthesis in dry seed tissue.

Two dehydrins were detected in dried seeds from the silver maple C1 seedlot (Fig. 5a), and three proteins were detected from those from the C2 seedlot (Fig. 5b), where the germination capacity after drying was twofold higher than the C1 seedlot (Fig. 1c). It was assumed that silver maple seeds were more viable because the 23-kDa dehydrin was present and the concentration of other dehydrins was relatively high. In fact, a positive correlation was observed between the ability of long-term stored beech (*Fagus sylvatica* L.) seeds to germinate and the 44-kDa dehydrin content (Kalemba and Pukacka 2008). Two bands with molecular masses of 34 and 35 kDa (Fig. 5) may represent the same protein for the same reason that is noted for the 23-kDa protein in the Norway maple seedlots. Beardmore and Whitle (2005) demonstrated that pretreatment with abscisic acid or tetcyclasis can stimulate the synthesis of the 44-and 32-kDa dehydrins, which could be related to the 46- and 35-kDa (Figs. 2, 4, 5) proteins that were identified in seeds of three *Acer* species.

Previous attempts to identify LEA proteins in *Acer* and other *recalcitrant* seeds produced inconclusive data (Finch-Savage and others 1994; Greggains and others 2000). However, Finch-Savage and others (1994) identified two identical dehydrins, with molecular masses of 18 and 23 kDa, in Norway maple and sycamore seeds. A 45-kDa dehydrin was detected in only embryonic axes of sycamore seeds, and one of the sycamore dehydrins was more concentrated in cotyledons than in embryonic axes. These findings suggest that *recalcitrant* seeds possess more dehydrin proteins than *orthodox* seeds, and *Acer saccharinum* seeds, which are more sensitive to desiccation than those of *A. pseudoplatanus*, contain even more of these proteins. This group also identified four dehydrins (47, 45, 22, and 20 kDa) in *A. saccharinum* embryonic axes and cotyledons. Greggains and others (2000) identified three dehydrins in Norway maple seeds; however, only one (a 20-kDa protein) was also present in sycamore seeds. By comparing the published data with the results presented in this study, it is hypothesized that all three *Acer* species contain at least three genes that encode dehydrin proteins and have approximate molecular masses of 46, 35, and 23 kDa. Small differences in the estimated molecular masses are probably due to the different prestained molecular mass markers that were used in the Western blot experiments. A 23-kDa dehydrin protein was clearly detected in the seeds of the examined *Acer* species (Figs. 2, 3, 4, 5), and the expression of two other dehydrins was highly complex. Two dehydrins (35 and 23 kDa) could be classified into the Y_nK_n- or K_n-type dehydrin family, where numerous representatives were broadly characterized and discussed (Rorat 2006; Battaglia and others 2008). The accumulation of the K_n subclass of dehydrin was regulated by cold stress, but the proteins did not accumulate in unstressed plants, as was shown for the *Arabidopsis* LTI30 K_6 dehydrin. The 46-kDa dehydrin undoubtedly contained a polyserine motif in the amino acid sequence (Fig. 6); therefore, as a highly phosphorylated protein, this dehydrin could play a role in the preservation of cell integrity during late embryogenesis and desiccation, as was suggested for the P-DHN-5 protein in wheat embryos (Brini and others 2007). Sunderlíková and others (2009) suggested that different classes of dehydrins could be involved in seed maturation processes and could respond to altered osmotic conditions. This conclusion was reached because they detected Y_nSK_n-type dehydrin expression during later stages of *recalcitrant* oak embryo development and two dehydrin genes that encoded putative K_n-type dehydrins in leaves of oak seedlings that had been exposed to desiccation.

Studies investigating both LEA and sHSPs in seeds have been described for *Brassic anapus* L. (Bettey and Finch-Savage 1998) and *Fagus sylvatica* L. (Kalemba and Pukacka 2008). One sHSP, with a molecular weight of 22 kDa, was observed in Norway maple (Fig. 7a), sycamore (Fig. 7b), and silver maple (Fig. 7c) seeds, and the concentration of this protein increased when seeds were dried. In beech seeds that had been stored for a long time, the increased level of the 22-kDa sHSP indicated high stability of the protein in dry seeds, and after storage, the protein had continuous protection activity (Kalemba and Pukacka 2008). In *Brassica* seeds, the highest level of the 18-kDa sHSP was positively correlated with the quality of the water-stressed seeds and with accelerated aging. Therefore, Bettey and Finch-Savage (1998) suggested that this chaperone protein might be involved in cell protection mechanisms. Alternatively, LEA proteins do not function as classical molecular chaperones (Kovacs and others 2008). This hypothesis is based on the observation that the expression of LEA proteins was not upregulated by heat shock factors (HSF) (Browne and others 2004); however, sHSPs could be activated by the dehydration-responsive element binding (DREB) family of transcription factors (Almoguera and others 2009). Conjointly overexpressing HaDREB2 and HaHSFA9 in sunflower seeds showed positive effects on seed longevity, whereas overexpressing HaDREB2 alone in seeds did not enhance their longevity. Interestingly, the functional interaction between HaDREB2 and HaHSFA9 was observed in seeds but not in vegetative tissues (Almoguera and others 2009). It is known that the removal of water from a cell can cause organelle crowding and irreversible structural changes, leading to the aggregation of macromolecules. Therefore, seeds that are prepared for storage are specifically exposed to dehydration after-effects. LEA proteins can help prevent protein aggregation due to desiccation (Goyal and others 2005), and the proposed mechanism for this action involves the amino acids of the Φ segment. This segment is

important in dehydrins for maintaining the disordered structure that allows the dehydrin protein to act as a molecular shield and prevent partially denatured proteins from interacting with one another (Hughes and Graether 2011). Prevention of protein aggregation is broadly realized by sHSPs by stabilizing the protein structure and restoring their native state (Halsbeck and others 2005). Both dehydrin proteins and sHSPs are essential for reducing the cellular damage that occurs during various dehydrative stresses (Kalemba and Pukacka 2007).

If the amounts of the protein extracts that were submitted to Western blot were, in fact, identical, the results of densitometry analyses suggest that the number and concentration of dehydrins and sHSPs in *orthodox* seeds (Figs. 2, 7a) are higher than in *recalcitrant* seeds (Figs. 3, 4, 7b, c). Protective proteins might be involved in preventing damage during desiccation in *orthodox* seeds and might assist in repair mechanisms after desiccation in *recalcitrant* seeds, but it is difficult to conclude that those proteins clearly define differences between the seed categories. Specifically, the variable profiles of dehydrins in sycamore and silver maple seeds and the remarkable differences of these profiles between years indicate that the attributes of *recalcitrant* seeds are difficult to unify. Dissimilar amounts of protective proteins in *orthodox* and *recalcitrant* seeds are most likely the result of the distinct gene expression regulation characteristics of each seed category. The potential modulation of dehydrin expression by environmental factors is possible; however, extended studies are needed to discover new links and come to a general conclusion for this hypothesis.

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