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ORIGINAL PAPER

Quantification of cellular protein expression and molecular features of group 3 LEA proteins from embryos of *Artemia franciscana*

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Abstract Late embryogenesis abundant (LEA) proteins are highly hydrophilic, low complexity proteins whose expression has been correlated with desiccation tolerance in anhydrobiotic organisms. Here, we report the identification of three new mitochondrial LEA proteins in anhydrobiotic embryos of *Artemia franciscana*, AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29. These new isoforms are recognized by antibody raised against recombinant AfrLEA3m, the original mitochondrial-targeted LEA protein previously reported from these embryos; mass spectrometry confirms all four proteins share sequence similarity. The corresponding messenger RNA (mRNA) species for the four proteins are readily amplified from total complementary DNA (cDNA) prepared from embryos. cDNA sequences of the four mRNAs are quite similar, but each has a stretch of sequence that is absent in at least one of the others, plus multiple single base pair differences. We conclude that all four mitochondrial LEA proteins are products of independent genes. Each possesses a mitochondrial targeting sequence, and indeed Western blots performed on extracts of isolated mitochondria clearly detect all four isoforms. Based on mass spectrometry and sodium dodecyl sulfate polyacrylamide gel electrophoresis migration, the cytoplasmic-localized AfrLEA2 exists primarily as a homodimer in *A. franciscana*. Quantification of protein expression for AfrLEA2, AfrLEA3m, AfrLEA3m_43, and AfrLEA3m_29 as a function of development shows that cellular concentrations are highest in diapause embryos and decrease during development to low levels in desiccation-intolerant nauplius larvae. When adjustment is made for mitochondria matrix volume, the effective concentrations of cytoplasmic versus mitochondrial group 3 LEA proteins are similar in vivo, and the values provide guidance for the design of in vitro functional studies with these proteins.

Keywords Intrinsically disordered proteins (IDPs) · Mitochondrion · Desiccation tolerance · Brine shrimp

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Introduction

When considering the ability to survive water stress, the most extreme examples are anhydrobiotic organisms, which can survive extended periods of almost complete desiccation (Keilin 1959; Crowe and Clegg 1973; Crowe and Madin 1974; Crowe and Clegg 1978; Clegg 2005; Watanabe 2006; Cornette and Kikawada 2011; Welnicz et al. 2011). In nature, anhydrobiotic organisms such as nematodes and tardigrades routinely experience dehydration down to 2 % tissue water (Crowe and Madin 1974; Alpert 2006), and the brine shrimp embryo can survive an even lower residual water content under aggressive experimental drying in the laboratory (Clegg et al. 1978; Hengherr et al. 2011a, b). As research on this topic progresses, it is becoming clear that desiccation tolerance relies on a number of different mechanisms and requires the stabilization of individual organelles in addition to cytosolic components (Pouchkina-Stantcheva et al. 2007; Tunnacliffe and Wise 2007; Hand and Hagedorn 2008; Atkin and Macherel 2009; Hand et al. 2011; Tripathi et al. 2012). The accumulation of low molecular weight organic solutes, such as trehalose, is often seen in desiccation tolerant organisms. These organic solutes aid in macromolecular protection at low water contents (Yancey et al. 1982; Yancey 2005). In addition to organic solutes, several types of protective macromolecules are correlated with desiccation tolerance, including late embryogenesis abundant (LEA) proteins and small stress proteins like *Artemia* P26, Hsp 21, and Hsp 22 (Clegg et al. 1994; Liang et al. 1997a, b; Willsie and Clegg 2001; Clegg

2005; Qiu and MacRae 2008a, b). Desiccation-tolerant embryos of *Artemia franciscana* possess a multitude of LEA proteins (Hand et al. 2007; Menze et al. 2009; Sharon et al. 2009; Chen et al. 2009; Warner et al. 2010, 2012; Wu et al. 2011). In the present study, we identify three new mitochondrial LEA proteins, AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29, and quantify protein expression for AfrLEA2, AfrLEA3m, AfrLEA3m_43, and AfrLEA3m_29 during diapause and development in *A. franciscana.* We also report evidence that cytoplasmic-targeted AfrLEA2 exists primarily as a homodimer in vivo.

To date, all LEA proteins described from animals have been assigned to group 3 (for classification scheme, see Wise 2003), with the exception of group 1 LEA proteins discovered in *A. franciscana* (Sharon et al. 2009; Warner et al. 2010; Wu et al. 2011; Marunde et al. 2013). Group 3 LEA proteins are predicted to have high alpha-helix content, but have been found experimentally to be unfolded when fully hydrated in aqueous solution (Goyal et al. 2003). Interestingly, Goyal et al. (2003) found that a group 3 LEA protein from an anhydrobiotic nematode adopted a α-helical structure upon desiccation, with a possible coiled-coil formation. Group 3 LEA proteins are characterized as being highly hydrophilic, intrinsically unstructured proteins with an overrepresentation of charged and acidic amino acid residues (Tunnacliffe and Wise 2007; Battaglia et al. 2008).

Various functions have been proposed for LEA proteins based on their natively unfolded structure and the correlation of gene expression to desiccation tolerance. Predicted physiological roles for LEA proteins include stabilization of sugar glasses (vitrified, noncrystalline structure in cells promoted by sugars like trehalose) (Wolkers et al. 2001; Hoekstra 2005; Shimizu et al. 2010), protein stabilization via protein–protein interaction or "molecular shield" activity (Tompa and Kovacs 2010; Chakrabortee et al. 2012), membrane stabilization (Tunnacliffe and Wise 2007; Tolleter et al. 2010), ion sequestration (Grelet et al. 2005), and formation of structural networks (Wise and Tunnacliffe 2004). Such networks of LEA proteins have been hypothesized to increase cellular resistance to physical stresses imposed by desiccation (Goyal et al. 2003).

Experimentally, LEA proteins prevent protein aggregation, protect enzyme function, and maintain membrane integrity during water stress (for reviews, see Tunnacliffe and Wise 2007; Hand et al. 2011; Hincha and Thalhammer 2012). However, the exact mechanisms for these protective abilities continue to be explored. Few studies attempt to rigorously estimate the effective cellular concentrations of LEA proteins (e.g., see excellent results for cotton seeds, Roberts et al. 1993). As a consequence, some functional roles projected from in vitro experiments may not be applicable in vivo because the concentrations used for in vitro characterization of LEA proteins are often arbitrary and may be unrealistic. In the present study, the titer of cytoplasmic-localized LEA protein (AfrLEA2) was 0.79±0.21 to 1.85±0.15 mg/g cellular water across development, and the combined mitochondrial-targeted LEA proteins (AfrLEA3m, AfrLEA3m_29, and AfrLEA3m_43) was roughly 1.2–2.2 mg/ml matrix volume for postdiapause embryos. Such estimates suggest that the effective concentrations of cytoplasmic versus mitochondrial group 3 LEA proteins are similar in vivo and provide guidance for the design of in vitro functional studies with these proteins.

Materials and methods

Cloning, expression, and antibody production for recombinant AfrLEA2 and AfrLEA3m

The original nucleic acid sequences for *Afrlea2* (GenBank accession no. EU477187) and *Afrlea3m* (GenBank accession no. FJ592175) cloned from *A. franciscana* embryos (Hand et al. 2007; Menze et al. 2009) were amplified from our existing *A. franciscana* cDNA library. Each gene was ligated into pET-30a (an expression vector with a T7 *lac* promoter; Novagen, Rockland, MA, USA) and then Rosetta™ 2(DE3) Singles™ Competent Cells (Novagen) were transformed with the genes according to the manufacturer's instructions. AfrLEA2 was expressed with an N-terminal 6X-His tag, and AfrLEA3m was expressed with a C-terminal 6X-His tag so as not to interfere with the mitochondrial localization sequence found at the N-terminus. Expression of recombinant LEA protein was induced by the addition of 1 mM IPTG for 2–3 h and confirmed by SDS-PAGE and protein staining with Coomassie Blue. Bacterial cells were pelleted by centrifugation (5,000×*g*, 15 min) at 4 °C and chemically lysed using Bugbuster® Protein Extraction Reagent (Novagen) in the presence of a protease inhibitor cocktail, P8849 (Sigma-Aldrich, St Louis, MO, USA). After removal of cellular debris by centrifugation, the cell lysate was subjected to affinity chromatography on a HisTrap™ FF crude column (GE Healthcare, Waukesha, WI, USA; 1 or 5 ml size, depending on experimental requirements). Affinity purification binding buffer contained 20 mM sodium phosphate, 0.5 M NaCl, and 20 mM imidazol, pH 7.5. A step elution was performed using an elution buffer containing 20 mM sodium phosphate, 0.5 M NaCl, and 0.5 M imidazol, pH 7.5. Flow rate was set at the maximum rate recommended by the manufacturer (1 ml/min for 1 ml column, or 5 ml/min for 5 ml column). Fractions containing recombinant protein were heat treated at 95 °C for 20 min followed by centrifugation (20,000×*g*, 30 min) to separate the soluble fraction. The soluble fraction was dialyzed overnight against the starting buffer for anion exchange (20 mM triethanolamine, 10 mM NaCl, pH 7.0). The sample was then applied to an anion exchange column (HiTrap™ Q FF; GE Healthcare). The elution buffer

contained 20 mM triethanolamine and 1 M NaCl, pH 7.0. The fractions containing pure recombinant protein, as assessed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and protein staining, were exchanged into LEA storage buffer (20 mM HEPES, 50 mM NaCl, pH 7.5) and concentrated using Amicon® Ultra Centrifugal filters (Ultracel®-10K; Millipore, Billencia, MA, USA). Antibodies were raised in chickens against recombinant AfrLEA2 and AfrLEA3m by Aves Labs, Inc. (Tigard, OR, USA).

Preparation of cDNA and sequencing of additional *Afrlea3m*-related genes

In extracts of mitochondria isolated from *A. franciscana*, four protein bands were identified with the AfrLEA3m antibody produced above (see "Results"). Consequently, we suspected that multiple mRNA species might be detected with cDNA prepared from *A. franciscana* embryos. Total RNA was isolated from diapause embryos using an RNeasy Midi kit (Qiagen, Valencia, CA, USA), and then a DyNAmo cDNA synthesis kit (New England Biolabs, Ipswich, MA, USA) was used for reverse transcription according to manufacturer's instructions. Primers for *Afrlea3m* amplified four products, which were cloned with a pENTR™/D-TOPO® Cloning Kit (Invitrogen, Carlsbad, CA, USA) as described in the manufacturer instructions. One Shot® TOP10 Chemically competent *Escherichia coli* (Invitrogen) were transformed with these genes. Direct colony PCR was performed to screen for transformed colonies. Colonies were identified that contained each of the four inserts, and a QIAprep 96 Turbo Miniprep Kit (Qiagen) was used to purify plasmid DNA from each. Sequencing was conducted with BigDye terminator chemistry and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular mass determination by SDS-PAGE

The molecular mass of recombinant and endogenous LEA proteins were determined by SDS-PAGE as described by Hames (1998). Briefly, the log of molecular mass for biotinylated protein standards (Cell Signaling Technology, Danvers, MA, USA) was plotted against relative migration distance (R_f) for the proteins after separation by SDS-PAGE. R_f was calculated as the migration distance of a protein divided by the migration distance of the dye front. The R_f values for LEA proteins were used to interpolate their molecular masses from the standard curves. The reported masses are the result of six separate measurements on three independent gels. Mitochondrial LEA samples were analyzed on 11 % acrylamide gels, and AfrLEA2 samples were analyzed on 7 % gels for optimal determination of size.

Mass spectrometry

In-gel trypsin digestion of proteins separated by SDS-PAGE was performed as described by Shevchenko et al. (2006). Briefly, bands of interest were excised from a Coomassie-stained SDS gel and destained in 100 μl of 100 mM ammonium bicarbonate/acetonitrile (1:1, vol/vol) for about 30 min. After destaining, 500 μl of 100 % acetonitrile was added to the destain mixture to dehydrate the gel pieces. The gel pieces were then transferred to 50 μl of a trypsin cocktail (13 ng protease/μl of a solution containing 10 mM ammonium bicarbonate and 10 % (vol/vol) acetonitrile) and incubated for 90 min to saturate the gel pieces with trypsin. Sequencing grade trypsin (catalogue no. V5111) was obtained from Promega (Fitchburg, WI, USA). Next 10–20 μl of 100 mM ammonium bicarbonate was added to the trypsin cocktail, and gel pieces were incubated overnight at 37 °C for complete protein digestion. Peptide products were extracted by adding 100 μl of extraction solution [5 % formic acid/acetonitrile (1:2, vol/vol)] to the trypsin cocktail and incubated on a shaker for 15 min at 37 °C. The liquid fraction containing the peptide digestion products was collected and dried in a vacuum centrifuge. Samples were submitted to the mass spectrometry Facility in the Department of Chemistry (Louisiana State University) and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS-MS) on a QSTAR XL quadrupole time-of-flight mass spectrometer (Applied Biosystems). The MS/MS data for each protein digest was submitted for a database search using Mascot from Matrix Sciences (Boston, MA, USA).

Preparation of protein extracts from diapause and postdiapause embryos

Diapause embryos were collected from the surface of the Great Salt Lake (Ogden, UT, USA) in fall 2011. Diapause embryos were maintained at ambient temperature in 1.25 M NaCl containing 200 U/ml nystatin, 50 mg/ml kanamycin, and 50 mg/ml penicillin-streptomycin; were protected from light. Prior to use, diapause embryos were rinsed and incubated in 35 ppt artificial seawater (Instant Ocean; Aquarium Systems, Mentor, OH, USA) at room temperature with shaking (110 rpm) for 4 days (Reynolds and Hand 2004) to allow hatching of any embryos that had broken diapause. Hatched nauplius larvae were removed, and intact diapause embryos were used for experiments. Postdiapause embryos of *A. franciscana* were obtained in the dry state from Great Salt Lake Artemia (Ogden, UT, USA; grade: laboratory reference standard) and stored at −20 °C. Prior to use, these dehydrated embryos were hydrated overnight in ice-cold 0.25 M NaCl. Embryos for the 0 h time point were processed immediately after hydration at 0 °C. Other embryos were transferred to

fresh 0.25 M NaCl at 23 °C and incubated with shaking (110 rpm) to promote pre-emergence development, and embryos were sampled at or 2, 4, 6, and 8 h. Prior to homogenization embryos were filtered and then blotted between two sheets of Whatman no. 41 filter paper to remove interstitial water. Blotting was performed according to Clegg (1974). To obtain nauplius larvae, hydrated embryos were incubated in 35 ppt artificial seawater for 24 h at 23 °C with shaking (110 rpm). Nauplius larvae were separated from unhatched embryos and shells, and then filtered and blotted.

For quantification of AfrLEA2 by Western blot (see below), 100 mg of embryos or 24 h nauplii were transferred directly into 1.9 ml of Laemmli sample buffer [62.5 mM Tris–HCl (pH 6.8), 2 % SDS, 10 % glycerol, and 5 % 2-mercaptoethanol (Laemmli 1970)] and homogenized in a ground glass homogenizer for 5–7 min. The homogenate was then heated at 95 °C for 5 min and centrifuged (10,000×*g*, 10 min) to remove the insoluble debris like shell and chitin fragments. In order to quantify the mitochondrial LEA proteins, it was necessary to first enrich these proteins in extracts because due to their mitochondrial location these proteins comprise a smaller percentage of total cellular protein compared to the cytosolic AfrLEA2. Accordingly, 200 mg of embryos (or 24 h nauplii) were instead homogenized into the nondenaturing LEA storage buffer described above. The homogenate was heated at 95 °C for 20 min and centrifuged (20,000×*g*, 30 min, 4 °C) to sediment the heat-insoluble fraction. The soluble supernatant, which contains only heat-stable macromolecules like LEA proteins, was retained and combined 3:1 with 4× Laemmli sample buffer. Protein concentration was obtained for all samples using a Lowry assay as described by Peterson (1977).

Preparation of isolated mitochondria

To obtain enriched preparations of mitochondrial LEA proteins and to confirm their mitochondrial localization, mitochondria were isolated as previously described (Kwast and Hand 1993; Reynolds and Hand 2004) from postdiapause embryos of *A. franciscana* that had been hydrated as described earlier and kept at 0 °C (i.e., hour 0 embryos). The final mitochondrial pellet was resuspended in 2 ml of homogenization medium and heat treated at 95 °C for 20 min. After centrifugation (20,000×*g*, 30 min, 4 °C), the heat-soluble fraction was diluted 1:2 with Laemmli sample buffer.

Western blot analysis

For *A. franciscana* samples 10 μg of total protein was loaded per lane on SDS acrylamide gels (4 % stacking gel and 11 % resolving gel) and electrophoresed for 80 min at 125 V in a Bio-Rad mini-Protean 3 cell. Proteins were then transferred to a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) in a transfer buffer containing 192 mM glycine, 20 % methanol, 0.025 % SDS, and 25 mM Tris; acceptable transfer was confirmed by staining the membranes with Ponceau S. Membranes were then blocked in a 5 % fat free dry milk solution for 1 h. Incubation with primary antibody raised against recombinant LEA protein (Aves Labs Inc.) was performed overnight at 4 °C, and then blots were washed for a total of 1 h with four changes of TBS-T (0.1 % Tween 20, 20 mM Tris–HCl, 500 mM NaCl, pH 7.6). Next, the membranes were incubated for 1 h at room temperature with the horse radish peroxidase-linked secondary antibody (goat anti-chicken; Aves Labs Inc.) and washed as above. Protein bands were visualized with LumiGLO chemiluminescent substrate (Cell Signaling Technology).

Quantity One Basic 4.6.9 software (Bio-Rad Laboratories) was used to quantify band intensities. Global background subtraction was applied to each image analyzed. These intensities were converted to ng LEA protein per band by comparing intensity values to a standard curve generated with known amounts of pure recombinant LEA protein. Experimental samples for AfrLEA2 were normalized to α-tubulin as a loading control before any further calculations were performed. Then, the values for nanograms protein/band were converted to mg protein/ml embryo water based on water content data previously published (Glasheen and Hand 1989). Mitochondrial LEA proteins were quantified similarly except they were not normalized to a housekeeper protein due to the heat treatment step required prior to Western blotting. Values for mitochondrial LEA proteins were reported as micrograms/gram wet tissue. Finally, to facilitate comparison to the effective in vivo concentration of AfrLEA2, mitochondrial LEA proteins were also expressed as mg protein/millimeter matrix volume. This calculation is based upon an estimated mitochondrial volume of 5 % of the total cell volume for postdiapause embryos of *A. franciscana* (Rees et al. 1989), and an estimate that matrix volume is about 50 % of total mitochondrial volume in the semicondensed/condensed states (Scalettar et al. 1991; Hackenbrock 1968).

Detection of glycoproteins in polyacrylamide gels

In-gel detection of glycoproteins was performed using the Periodic acid–Schiff (PAS) method. Schiff's fuchsin-sulfite reagent was obtained from Sigma-Aldrich, and detection was performed as described by the supplier. Briefly, gels were incubated in fixative solution (40 % ethanol and 7 % acetic acid) for 1.5 h with four changes of solution and then left in the fixative overnight. Afterwards, the fixative solution was again refreshed twice, each followed by a 30-min incubation. Oxidation of glycoprotein bands was accomplished by immersing gels in a solution containing 1 % periodic acid and 3 % acetic acid for 60 min. Gels were then washed ten times (10 min each) to remove traces of periodic acid before incubation in Schiff's reagent for 60 min in the dark. Stained gels

were washed in a solution with 0.58 % potassium disulfite and 3 % acetic acid to remove any background.

Results

Properties of AfrLEA2 in *A. franciscana* embryos

As previously reported, AfrLEA2 is a group 3 LEA protein (38.9 kDa) that is predicted to reside in the cytoplasmic compartment (Hand et al. 2007). This predicted location was confirmed in HepG2 cells transfected with the green fluorescent protein-tagged protein (Li et al. 2012). Recombinant AfrLEA2 has a total molecular mass of 43.1 kDa (38.9 kDa plus 4.2 kDa for a 6×-His tag and associated sequence) but migrates on SDS gels at a calculated mass of 49.3±0.9 (mean±SD; $n=3$) (Fig. 1a). This higher apparent molecular mass with SDS-PAGE may be explained by the observation that some intrinsically disordered proteins have a reduced binding for SDS and therefore frequently exhibit decreased migration on SDS gels (Tompa 2002). Based on the migration of recombinant AfrLEA2, we expect endogenous AfrLEA2 to migrate at about 44 kDa. Surprisingly, when *A. franciscana* extracts are electrophoresed on an 11 % polyacrylamide gel and probed with anti-AfrLEA2 polyclonal antibody, a protein band is detected around 75 kDa (data not shown). Upon closer examination with a 7 % polyacrylamide gel to provide better resolution of larger proteins, three bands are discernible, with calculated apparent molecular masses of 82.0±0.4, 75.3±1.0, and 72.7±0.5 kDa (means±SD; $n=3$) (Fig 1a). For most developmental stages of *A. franciscana* where AfrLEA2 is expressed, the 82 kDa form is predominant. Based on the molecular mass and further evidence below, we suggest that the 82 kDa protein is a dimer of the 38.9 kDa AfrLEA2, and the smaller bands may be degradation products or even processed forms of AfrLEA2 (cf. Goyal et al. 2005a; Kikawada et al. 2006).

Oligomers of LEA proteins resistant to SDS dissociation have been previously documented by PAGE (Goyal et al. 2003), and we detected apparent dimers and trimers of purified recombinant AfrLEA2 by SDS-PAGE with anti-AfrLEA2 antibodies (Fig 1a; 97.6 and 137.5 kDa), which supports the ability of the protein to form oligomers. Apparent multimers of recombinant AfrLEA2 are also visualized using an anti-6×-His antibody (data not shown), which suggests the multimers are not nonspecific products that cross-react with AfrLEA2 antibody. While only small numbers of cytoplasmic proteins are typically glycosylated, in-gel staining with PAS reagent did not indicate AfrLEA2 to be glycosylated, based on the absence of PAS-positive bands of appropriate size in embryo extracts enriched for LEA proteins by heat purification. In-gel trypsin

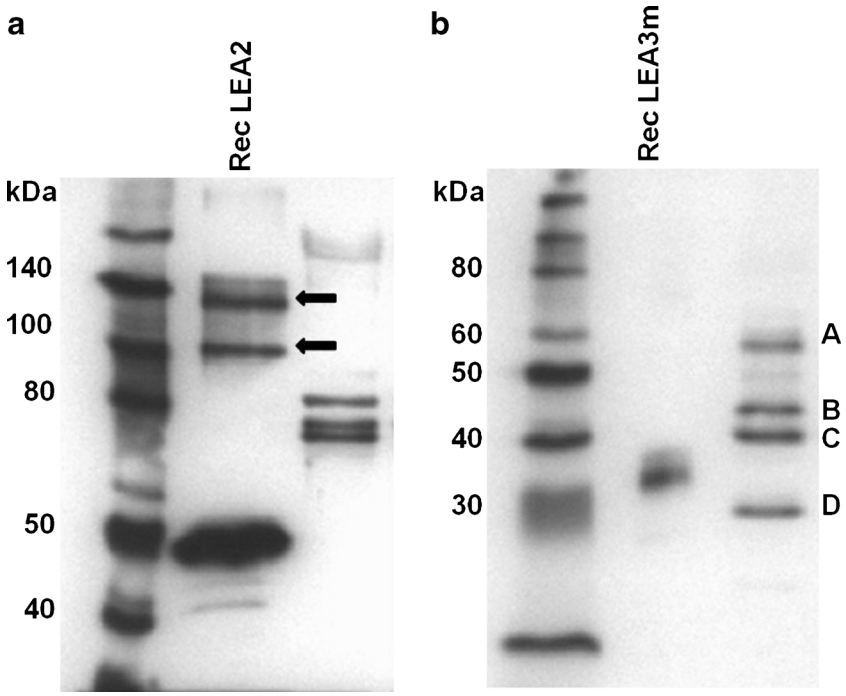

Fig. 1 Western blot analyses of purified recombinant proteins and various extracts from *Artemia franciscana*. **a** *Lane 1* was loaded with molecular mass standards, *lane 2* with 1 μg of recombinant AfrLEA2 protein, and *lane 3* with 10 μg protein of an extract prepared from diapause embryos. With the diapause extract, AfrLEA2 antibody reveals three bands on a 7 % polyacrylamide gel with apparent molecular masses of 82.0±0.4, 75.3±1.0, and 72.7±0.5 kDa. Arrows indicate dimers and trimers formed by recombinant AfrLEA2. **b** *Lane 1* was loaded with molecular mass standards, *lane 2* with 50 ng of recombinant AfrLEA3m protein, and *lane 3* with extract prepared from mitochondria isolated from postdiapause embryos (hour 0). AfrLEA3m antibody detects four bands (labeled **a**–**d**) with apparent molecular masses of 62.3±0.4, 48.6 ±1.4, 43.5±0.1, and 32.4±1.1 kDa in extracts prepared from isolated mitochondria

digests were performed on gel slices from the regions where the dimer and monomer migrate, and the peptides were analyzed by mass spectrometry (LC-MS-MS). One or more peptide fragments from both areas were found to share sequence identity to bona fide AfrLEA2 based on robust scores (Table 1). Finally, with *Afrlea2*-specific primers, PCR amplification was performed on cDNA prepared from diapause embryos. Only a single 1.1 kb product was generated, which is the expected size of mRNA encoding for AfrLEA2 (364 amino acids; Fig. 2). The combined evidence indicates that the predominant 82 kDa protein visualized on Western blots with AfrLEA2 antibody is a dimer of the 38.9 kDa AfrLEA2.

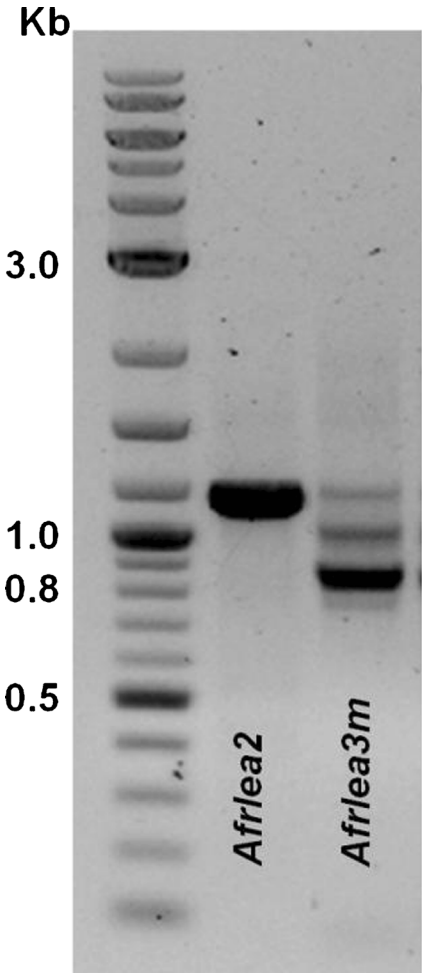

Fig. 2 PCR products amplified with cDNA prepared from diapause embryos of *A. franciscana*. *Lane 1* was loaded with a DNA ladder (kb) and *lanes 2* and *3* with products from PCR reactions as indicated. Primers designed against *Afrlea2* yielded a single product of approximately 1.1 kb (*Afrlea2* lane). Primers designed against *Afrlea3m* amplified four products that migrate at 1.2, 1.0, 0.9, and 0.7 kb (*Afrlea3m* lane)

Multiple independently encoded variants of AfrLEA3m

AfrLEA3m is a mitochondrial LEA protein with a deduced molecular mass of 34.1 kDa or 30.9 kDa if the targeting sequence were to be cleaved (Menze et al. 2009). However, because the cleavage site is only predicted by bioinformatics and has not been confirmed experimentally, we will use the full-length deduced molecular mass when referring to AfrLEA3m. The mitochondrial location of AfrLEA3m was previously confirmed in HepG2 cells stably transfected with *Afrlea3m* (Li et al. 2012). Western blot analysis of mitochondria isolated from 0 h postdiapause embryos reveals four distinct bands when probed with antiserum against AfrLEA3m (Fig. 1b). In-gel trypsin digests of all four bands were analyzed by mass spectrometry (LC-MS-MS) and found to share sequence identity to AfrLEA3m in three or more regions with a lowest combined score of 177 (Table 2).

With *Afrlea3m* primers and cDNA template prepared from diapause embryos, PCR amplification generated four products (Fig. 2). In order to gain more insight into the nature of these mRNAs, each of the four bands amplified from cDNA was cloned and sequenced. The protein encoded by the 924 bp cDNA is identical to AfrLEA3m that was previously reported (Menze et al. 2009). Like AfrLEA3m, all three newly identified proteins (AfrLEA3m_47, AfrLEA3m_43, and AfrLEA3m_29; suffixes indicate masses deduced from cDNA sequence) possess mitochondrial targeting sequences and thus are predicted to localize to the mitochondrion with high probability (Target P, MitoProt II, and Predator). This prediction is supported by the fact that Western blots performed with isolated mitochondria clearly reveal all four isoforms of AfrLEA3m (Fig. 1b). The deduced protein sequences for the three new isoforms are highly

Table 1 Mass spectrometry confirms that the two molecular mass forms of AfrLEA2 share sequence similarity with the bona fide purified protein

AfrLEA2 monomer		AfrLEA2 dimer	
Score	Peptide	Score	Peptide
47	K.SISDAAYFTGK.G	83	K.GIGETVKADADVVEGMASTGYEK.L
56	K.INAIQTPEEMDHER.L		
101	K.GIGETVKADADVVEGMASTGYEK.L		

Mascot ions scores are −10*log(P), where P is the probability that the observed match is a random event. Individual ions scores, or combined scores where multiple peptides are identified from a single protein, >60 indicate identity or extensive homology ($p < 0.05$)

Table 2 Mass spectrometry confirms that the four protein isoforms recognized by antibody raised against recombinant AfrLEA3m share sequence similarity to bona fide AfrLEA3m

AfrLEA3m_29		AfrLEA3m_43	
Score	Peptide	Score	Peptide
55	K.ESWESLKDTAK.Q	53	K.AKGEYDPEYPLSSSMK.A
36	K.LSWEDTKETYK.E	53	K.ESVSSTSSEAQNRGESMYGK.T
41	K.AKGEYDPEYPLSSSMK.A	71	K.SAYESIKESVSSTSSEAQNR.G
41	K.ESVSSTSSEAQNRGESMYGK.T		
73	K.SAYESIKESVSSTSSEAQNR.G		
AfrLEA3m		AfrLEA3m_47	
Score	Peptide	Score	Peptide
60	K.ESWESLKDTAK.Q	54	K.AKGEYDPEYPLSSSMK.A
31	K.LSWEDTKETYK.E	81	K.GGSGFNQITPEQTENMK.G
76	K.GEYDPEYPLSSSMK.A	57	K.ESVSSTSSEAQNRGESMYGK.T
39	K.AKGEYDPEYPLSSSMK.A	69	K.SAYESIKESVSSTSSEAQNR.G
71	K.GGSGFNQITPEQTENMK.G		
56	K.ESVSSTSSEAQNRGESMYGK.T		
74	K.SAYESIKESVSSTSSEAQNR.G		

Mascot ions scores are $-10*\log(P)$, where P is the probability that the observed match is a random event. Individual ions scores, or combined scores where multiple peptides are identified from a single protein, >60 indicate identity or extensive homology ($p<0.05$)

hydrophilic as determined by Kite and Doolittle hydropathy plots (data not shown). The sequences for all four cDNAs are very similar, but each has a stretch of sequence that is absent in at least one of the others (Fig. 3). In addition, *Afrlea3m_43* has five single nucleotide changes scattered across its sequence that do not match the other three cDNAs (data not shown), and *Afrlea3m_47* and *Afrlea3m_43* have three single nucleotide differences in the section of sequence shared by these two variants (Fig. 3, yellow bar). Thus, we conclude that these mRNA species are independently encoded, but closely related variants, of *Afrlea3m*.

As anticipated based on the earlier discussion, the four mitochondrial isoforms of AfrLEA3m migrate on SDS gels with larger apparent molecular masses than predicted. The apparent molecular masses for AfrLEA3m_47, AfrLEA3m_43, AfrLEA3m, and AfrLEA3m_29 are 62.3±0.4, 48.6±1.4, 43.5±0.1, and 32.4±1.1 kDa (mean±SD; $n=3$), respectively. Mass spectrometry data supports the identity of the protein that migrates in embryo extracts at 43.5 kDa (Fig. 1b, c) as AfrLEA3m. Recombinant AfrLEA3m (34.9 kDa with 6X-His tag) migrates with an apparent molecular mass of 35.8±0.3 kDa (mean±SD; $n=3$); because AfrLEA3m, when naturally expressed in embryos, migrates at 43.5 kDa, we cannot exclude the possibility of posttranslational modification (Irar et al. 2006; Alsheikh et al. 2003). Mass spectrometry rules out the possibility that band C corresponds to AfrLEA3m_29 or AfrLEA3m_43. Although mass spectrometry does not eliminate AfrLEA3m_47, it is highly unlikely that band C corresponds to this protein due to the large difference in size.

Protein expression of AfrLEA2 during development

By comparing band intensities to a standard curve created using recombinant AfrLEA2 (Fig. 4), we were able to estimate the quantity of AfrLEA2 (subforms combined) present in each sample. These values were then converted to mg LEA protein per ml embryo water. Because AfrLEA2 is a cytoplasmic-localized protein, this concentration unit provides a meaningful estimate of its effective titer in vivo. AfrLEA2 is most abundant in diapause and decreases throughout development to undetectable levels in 24 h nauplius larvae (Figs. 4a and 5). This pattern is in agreement with the mRNA expression profile

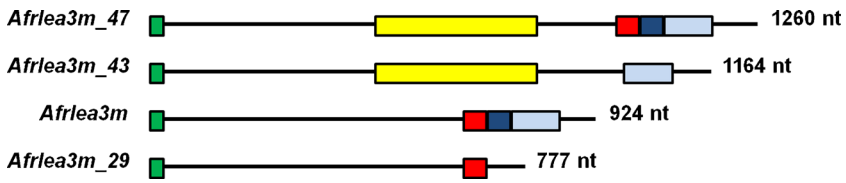

Fig. 3 Sequence comparisons for the four cDNA sequences amplified with primers designed against *Afrlea3m*. The *green boxed regions* (nucleotide 1–84) indicate the mitochondrial leader sequence that is shared by all four cDNAs. Each of the other four boxed regions (*yellow*, *red*, *light blue*, and *dark blue*) indicate stretches of virtually identical sequence that are present in some of the cDNAs but absent in at least one. AfrLEA3m_47: *yellow box* nucleotide 466–801, *red box* nt 989–1036, *dark blue box* nt 1037–1084, and *light blue box* nt 1085–1183. AfrLEA3m_43: *yellow box* nt 466–801 and *light blue box* nt 989–1087. AfrLEA3m: *red box* nt 653–700, *dark blue box* nt 701–748, and *light blue box* nt 749–847. AfrLEA3m_29: *red box* nt 653–700. None of the mRNA variants contain sequence that was not shared by at least one other isoform. *Solid black lines* indicate virtually identical sequence shared by all four cDNAs

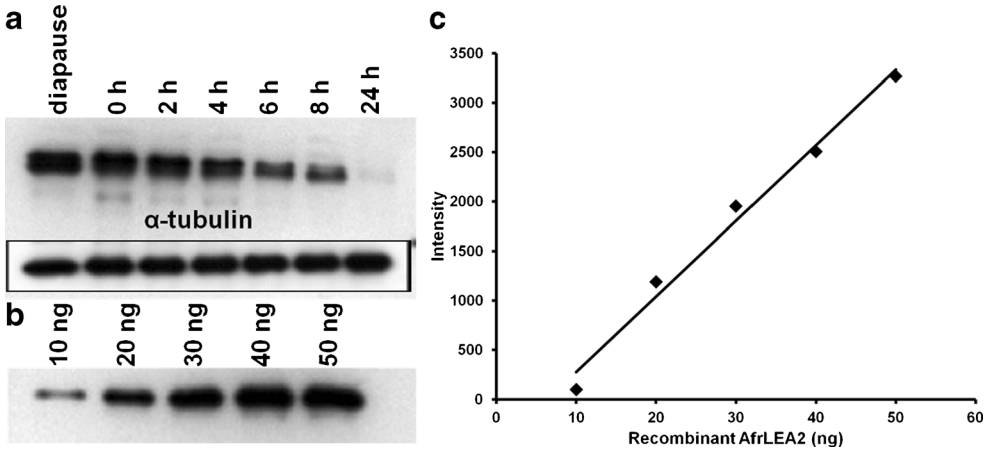

Fig. 4 Quantification of AfrLEA2 protein in extracts of *A. franciscana* by Western blot analysis. **a** Expression levels for AfrLEA2 are shown at various stages of the life cycle [diapause; pre-emergence development (hours 0, 2, 4, 6, and 8); and nauplius larvae (24 h)]. AfrLEA2 is most abundant in diapause and decreases throughout development to undetectable levels in nauplius larvae. α-Tubulin is included as a loading control for each time point. **b** Concentration dependency of recombinant AfrLEA2 as measured with anti-AfrLEA2 antibody. **c** Standard curve for recombinant AfrLEA2 (R^2=.986)

reported for *Afrlea2* (Hand et al. 2007) and further supports the role for LEA proteins in desiccation tolerance in *A. franciscana*, a physiological feature that disappears beginning at the larval stage. Based on these results there are 1.85± 0.15 mg (mean±SD; n=3) of AfrLEA2 per milliliter embryo water in diapause embryos (Fig. 5) or 5.05 μg of AfrLEA2 per milligram total embryo protein.

Quantification of protein expression for AfrLEA3m, AfrLEA3m_43, and AfrLEA3m_29 during development

As described above, a standard curve (Fig. 6) was used to determine the concentrations of AfrLEA3m, AfrLEA3m_43, and AfrLEA3m_29 present in each sample. The low amount of expressed AfrLEA3m_47 made it problematic to quantify. Because these three proteins are localized to the mitochondrial compartment, the concentrations of each mitochondrial LEA were initially expressed as micrograms protein per gram wet tissue (Fig. 7). There were significant decreases in content for each of the three proteins from diapause through pre-emergence development (0–8 h), and then even further decreases occurred in 24 h nauplius larvae (Fig. 7). These trends in protein expression mirror the mRNA expression data reported for *Afrlea3m* (Menze et al. 2009).

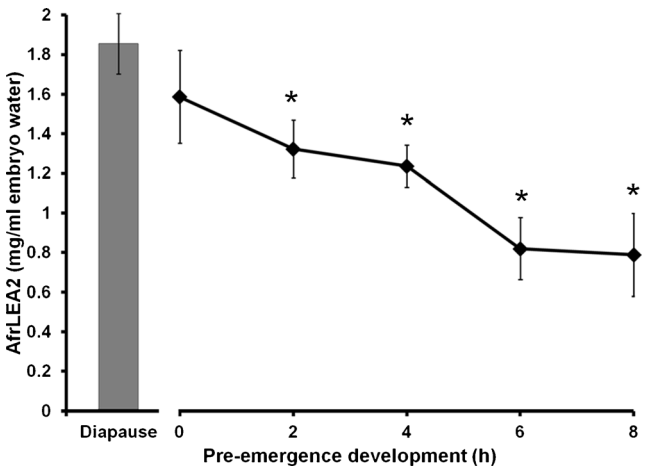

Fig. 5 AfrLEA2 concentrations from diapause through 8 h of pre-emergence development. All values were normalized to α-tubulin. *Asterisks* indicate that the means are statistically different from diapause values (one-way-ANOVA, Tukey, P<0.05). Conversion of AfrLEA2 concentrations to "per milliliter embryo water" is based on water content data previously published (Glasheen and Hand 1989)

Finally, to estimate an effective in vivo concentration, the amount of combined mitochondrial-targeted LEA proteins (AfrLEA3m, AfrLEA3m_29, and, AfrLEA3m_43) was also expressed as milligrams protein/milliliter mitochondrial matrix volume. For postdiapause embryos, this value is approximately 1.2 mg protein/milliliter matrix volume. Considering that the mitochondrial density in cells is comparable between diapause and postdiapause embryos (Reynolds and Hand 2004), the value would be approximately 2.2 mg/ml during diapause. Interestingly, such estimates suggest that the effective concentrations of cytoplasmic versus mitochondrial group 3 LEA proteins are comparable in vivo and provide guidance for the design of in vitro functional studies with these proteins.

Discussion

In the present study, we have identified three new mitochondrial LEA proteins in *A. franciscana* and characterized the protein expression levels for four group 3 LEA proteins throughout *A. franciscana* development. The four mitochondrial LEA proteins studied here share similar sequence identity, but contain multiple single base pair differences, so we

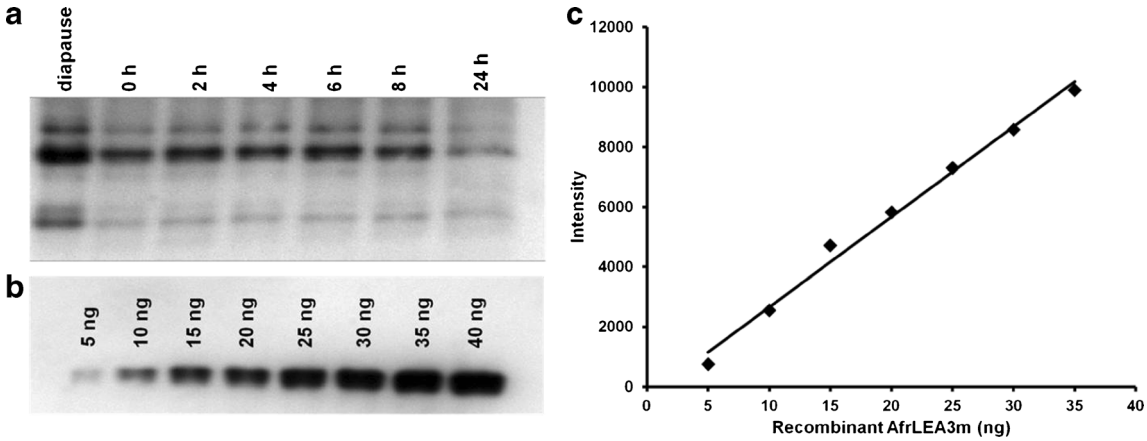

Fig. 6 Quantification of mitochondrial LEA proteins by Western blot analysis in heat-treated extracts of *A. franciscana*. **a** Expression levels for mitochondrial LEA proteins are shown at various stages of the life cycle [diapause, pre-emergence development (hours 0, 2, 4, 6, and 8), and nauplius larvae (24 h)]. AfrLEA3m isoforms are most abundant in diapause and decrease throughout development to the lowest levels observed, which are found in nauplius larvae. Equal amounts of total protein in extracts were loaded for each time point. **b** Concentration dependency of recombinant AfrLEA3m as measured with anti-AfrLEA3m antibody. **c** Standard curve for recombinant AfrLEA3m (R^2=.99)

predict that these mRNA are encoded by separate genes. We have previously reported mRNA expression for AfrLEA2 (Hand et al. 2007) and AfrLEA3m (Menze et al. 2009) to be highest in desiccation tolerant stages (diapause and postdiapause embryos) when compared to desiccation-sensitive nauplius larvae. The protein expression patterns reported in this study are in agreement with the mRNA expression and provide further evidence that LEA proteins play a role in desiccation tolerance. Finally, we have also experimentally measured physiologically relevant quantities of a cytoplasmic (AfrLEA2) and three mitochondrial (AfrLEA3m_43, AfrLEA3m, and AfrLEA3m_29) LEA proteins across development in *A. franciscana*. Values of this type are useful to more accurately evaluate whether concentration-dependent properties identified for LEA proteins in vitro are relevant for in vivo settings.

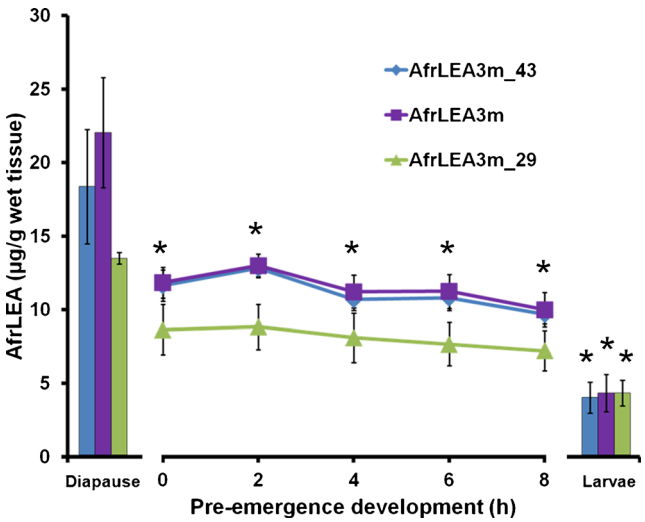

Fig. 7 Protein concentrations of AfrLEA3m_43, AfrLEA3m, and AfrLEA3m_29 for diapause, 0–8 h of pre-emergence development, and 24 h nauplius larvae. For each LEA protein, the *asterisks* indicate that the means for pre-emergence and larval stages are statistically different from their respective diapause value (one-way-ANOVA, Tukey, P<0.05). AfrLEA3m_47 was too faint to reliably quantify across development

Our results provide evidence that endogenous AfrLEA2 exists primarily as a dimer in *A. franciscana* embryos. The presence of SDS-resistant oligomers have been previously reported for LEA proteins and other hydrophilic proteins (Goyal et al. 2003; Bahrndorff et al. 2009; Maskin et al. 2007), but this is the first report to our knowledge of a LEA protein existing primarily as a dimer in vivo. In addition to molecular mass, key evidence for the 82-kDa dimer includes the amplification with *Afrlea2*-specific primers of only a single PCR product and that this product is of the correct size for a mRNA encoding the 38.9 kDa monomer. Further, a few bases upstream of the *Afrlea2* gene is sequence for a translational stop codon. Mass spectrometry of the dimer supports sequence similarity to the monomer. At high concentrations, our purified recombinant AfrLEA2 will form dimers and trimers in vitro that are resistant to SDS dissociation. The smaller bands (75.3 and 72.7 kDa) recognized by the anti-AfrLEA2 antibody could be processed forms of the AfrLEA2 dimer, as reported by Goyal et al. (2005a) for a group 3 LEA protein (AavLEA1) from the nematode *Aphelenchus avenae*. These authors provide evidence for nonrandom cleavage that could increase the specific activity of the LEA protein, whereby two shorter proteins are more effective molecular shields than one larger one. Alternatively, intrinsically disordered proteins, such as LEA proteins, are susceptible to random degradation due to their unstructured nature (Receveur-Brechot et al. 2006; Uversky and Dunker 2010).

Unlike the results for *Afrlea2*, four distinct bands were amplified from cDNA of diapause embryos with primers

designed for *Afrlea3m*, which indicates that the four proteins identified by our anti-AfrLEA3m antibody are encoded by individual mRNA species. While general architectural features of the coding sequences display similarities that include sequence identity at their N-termini (Fig. 3), the multiple single-nucleotide differences distributed across the sequences preclude splice variants as an explanation. Although not absolutely proven experimentally, we suggest that these four mRNAs are products of separate, independent genes. A similar case is seen in rotifers, where two LEA mRNAs are very similar but arise from two individual genes on different chromosomes (Pouchkina-Stantcheva et al. 2007). In addition, very similar variants of group 1 LEA proteins have been documented in *A. franciscana* and attributed to independent genes (Sharon et al. 2009; Warner et al. 2010; Warner et al. 2012; Marunde et al. 2013).

All four of the mitochondrial AfrLEA3m proteins migrate with an apparent molecular mass 1.12–1.34 times higher than their deduced molecular masses. This result is well within the range of SDS migration discrepancy reported for intrinsically disordered proteins by Tompa (2002). Interestingly, mass spectrometry data indicate the protein that migrates with an apparent molecular mass of 43.5 kDa is indeed AfrLEA3m, for which the purified recombinant form migrates at 35.8 kDa (deduced mass of 34.9 kDa with 6X-His tag). This disparity in migration introduces the possibility that at least one LEA protein may be posttranslationally modified as natively expressed in *A. franciscana*. We do not have recombinant versions of the other three mitochondrial LEAs in this study with which to directly compare against their natively expressed counterparts. It is appropriate to note that AfrLEA3m expressed in human HepG2 cells migrates similarly to the recombinant form of AfrLEA3m (Li et al. 2012). Consequently, if AfrLEA3m is in fact posttranslationally modified by *A. franciscana*, then this capacity appears to be absent in human cells.

As research into the function of LEA proteins continues, it is important to consider their endogenous cellular titer, and how LEA protein concentration relates to proposed in vivo functions. Considering that an individual LEA protein family can represent up to 3.86 % of total cytosolic protein in plant seeds (Roberts et al. 1993) and that most organisms express a multitude of LEAs, it is becoming apparent that LEA proteins can embody a large proportion of total cellular protein. We have shown that AfrLEA2, one of the multiple cytosolic LEA proteins expressed in *A. franciscana*, has a cellular concentration of 1.85 mg protein/ml cell water, and three of the known mitochondrial LEAs from *A. franciscana* have a combined concentration of 2.2 mg protein/ml mitochondrial matrix volume. These values can be used to re-evaluate previous predictions for LEA protein function. For example, Tolleter et al. (2007) predict that LEAM, a mitochondrial protein expressed in pea seeds, provides protection to the inner mitochondrial membrane during desiccation. According to their calculations, LEAM would need to represent about 0.6 % of total matrix protein in order to provide protection to about one third of the inner membrane surface (an estimate of the protein-free area). With total matrix protein in the range of 400 mg/ml, this predicted amount of 2.4 mg/ml LEAM is not unreasonable based on our estimation that three mitochondrial LEA proteins from *A. franciscana* embryos are present at a combined concentration of 2.2 mg/ml.

Another important consideration is the design and interpretation of in vitro studies used to attribute functional characteristics to various LEA proteins. Knowledge of the endogenous titers of LEA proteins is fundamental because it can be used as a starting point for estimating mass ratios of LEA protein to target molecule in vivo. The ability of LEA proteins to stabilize sugar glasses (Wolkers et al. 2001), model membranes (Tolleter et al. 2010), and proteins (Goyal et al. 2005b) have been investigated in vitro at mass ratios of LEA protein to target as high as 2:1, 1:3, and 40:1, respectively. Although there may be situations where the use of endogenous levels of either LEA proteins or target molecules are not practical (due to cost or availability), it is important to take the cellular titers of LEA proteins into consideration when interpreting results, especially when translating functional characteristics of LEA proteins from in vitro to in vivo conditions. Furthermore, an open question exists as to why the presence of LEA proteins without protective sugars is sufficient for desiccation tolerance in some anhydrobiotic species, while in other tolerant animals, high concentrations of glass-forming sugars (e.g., trehalose) are preferentially accumulated during drying together with LEA proteins (cf. Hand et al. 2011). Perhaps, the absolute cellular titer of LEA proteins expressible in a given cell type/organism governs the apparent need for trehalose.

Several reports now indicate that a multitude of LEA proteins can be expressed in a given anhydrobiotic organism, which brings into question why it is necessary for one organism to express so many different LEA variants. Differential subcellular targeting may be one reason for the presence of so many LEA proteins in a single organism. Animal LEA proteins have been found commonly in the cytoplasm (for review see Hand et al. 2011) as well as multiple subcellular locations including the mitochondrion (Grelet et al. 2005; Menze et al. 2009; Warner et al. 2010; Warner et al. 2012), nucleus (Warner et al. 2012), endoplasmic reticulum, golgi, and extracellular space (Tripathi et al. 2012). In addition to differential subcellular targeting, LEA proteins have been shown to stabilize different classes of macromolecules during water stress (for, e.g., see Pouchkina-Stantcheva et al. 2007). Considered together, it is possible that differential subcellular targeting and the ability of individual LEA proteins to stabilize different types of macromolecules may explain the necessity for multiple LEAs within a single organism.

In conclusion, our results for differential ontogenetic expression of LEA proteins support their involvement in

desiccation tolerance in *A. franciscana*, and we provide physiological protein concentrations for LEA proteins in two different cellular compartments, the cytoplasm and the mitochondrion. Appreciating the cellular titers of LEA proteins in different organisms is important to further our understanding of how these proteins function, and can be used as a guide to design future in vitro experiments. Finally, we have identified three new mitochondrial LEA proteins that add to the already substantial list of LEA proteins expressed by *A. franciscana* (Hand et al. 2007; Menze et al. 2009; Sharon et al. 2009; Warner et al. 2010, 2012; Wu et al. 2011; Chen et al. 2009), which arguably should be considered an animal extremophile (Clegg 2011).

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