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Life Without Water: Expression of Plant LEA Genes by an Anhydrobiotic Arthropod

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ABSTRACT Anhydrobiotic animals protect cellular architecture and metabolic machinery in the dry state, yet the molecular repertoire supporting this profound dehydration tolerance is not fully understood. For the desiccation-tolerant crustacean, *Artemia franciscana*, we report differential expression of two distinct mRNAs encoding for proteins that share sequence similarities and structural features with late-embryogenesis abundant (LEA) proteins originally discovered in plants. Bioinformatic analyses support assignment of the LEA proteins from *A. franciscana* to group 3. This eucoelomate species is the most highly evolved animal for which LEA gene expression has been reported. It is becoming clear that an ensemble of micromolecules and macromolecules is important for establishing the physical conditions required for cellular stabilization during drying in nature. *J. Exp. Zool. 307A:62–66, 2007.*

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It is a biological truism that environmental stresses that impact the activity of cellular water pose a threat to life (Somero, '92). For centuries scientists have been intrigued by exceptional animals that can survive the loss of virtually all cellular water for prolonged periods (Leeuwenhoek, 1702; Crowe and Clegg, '73). The mechanisms by which these anhydrobiotic animals protect cellular architecture and metabolic machinery in the dry state are not only of biological interest but also of biomedical importance for cell stabilization (Crowe et al., 2005; Elliott et al., 2006). For a desiccation-tolerant arthropod, we report herein the differential expression of two distinct mRNAs encoding for proteins that share strong sequence similarities and structural features with late-embryogenesis abundant (LEA) proteins originally discovered in plants.

Intracellular sugar glasses like those formed with trehalose or other carbohydrates during drying (Hoekstra, 2005 and references therein) provide protection during water stress in animals and plants. Hydrophilic LEA proteins (hydrophilins) seen for example in seeds, pollen, and the resurrection plant are receiving considerable interest because their intracellular accumulation is tightly correlated with acquisition of desiccation tolerance, and recent in vitro data indicate their capacity to stabilize other proteins during drying (Grelet et al., 2005). Embryos of the brine shrimp *Artemia franciscana* have served as an important model species for studies of animal desiccation, and evidence from this organism has underscored the role of trehalose and small stress proteins in anhydrobiosis and other environmental stresses (Crowe et al., '84; Warner et al., 2004; Clegg, 2005; Crowe et al., 2005). In this study, we investigated whether or not expression of other desiccation-associated proteins might be found in the developmental stages of the brine shrimp that exhibit survival in the dried state.

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MATERIAL AND METHODS

Animals

Encysted embryos of the brine shrimp, *A. franciscana* Kellog (Great Salt Lake population), were either obtained in the dehydrated state (post-diapause) from Sanders Brine Shrimp Co. (Ogden, UT) or collected from the surface of the Great Salt Lake in the hydrated state (diapause) (Covi and Hand, 2005). Embryo viability and preservation of the diapause state during storage were evaluated as previously described (Reynolds and Hand, 2004).

Sequencing of cloned LEA genes

DNA inserts were sequenced directly from plasmids isolated from bacterial clones picked from a full-length unidirectional cDNA Library (Lambda Uni-ZAP XR Vector). The library was prepared from poly-A mRNA purified and pooled from active and diapause stage embryos. Sequencing utilized BigDye terminator chemistry and an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Primer walking was used to insure full-length sequence was obtained. Sequences were assembled using Sequencher software (Gene Codes Co., Ann Arbor, MI).

Isolation of total RNA and preparation of cDNA for quantitative PCR

Post-diapause embryos were hydrated and incubated at room temperature in medium equilibrated with air as described previously (Covi and Hand, 2005). Briefly, after the desired time periods of incubation, RNA extractions were performed with an RNeasy Midi kit (Qiagen, Valencia, CA) as per the manufacturer's instructions for animal tissues. For samples of larvae (24 hr), swimming nauplii were separated from the shed chorion. Diapause embryos were incubated for 4 d as previously reported (Reynolds and Hand, 2004) to allow individuals not in diapause to hatch; larvae and empty shells were removed. The concentration of RNA in each sample was determined spectrophotometrically (A_{260}). AMV reverse transcriptase (Promega, Madison, WI) and a $dT_{25}V$ primer were employed to reverse transcribe poly(A+) mRNA from total RNA.

Real-time quantitative PCR

Analyses of cDNA from various developmental stages of *A. franciscana* were performed using real-time quantitative polymerase chain reaction (rtqPCR) with an iCycler (Bio-Rad Laboratories, Hercules, CA) and SYBR Green. PCR reaction mixes were prepared by using the IQ SYBR Green Supermix (Bio-Rad Laboratories) with a total reaction volume of 25 μl. Cycling parameters were 3 min at 95°C and then 50 cycles at 95°C (15 sec) and 56°C (30 sec), followed by a melting curve analysis. Each developmental stage was evaluated with 4–6 independent experiments, each with three nested replicates, and with reference dye normalization. The cycle threshold value (Ct) determined with the manufacturer's software and used for analyses, and all ΔCt values were normalized to the expression of a bona fide housekeeping gene, α-tubulin (Zheng et al., '98), after determination of the PCR efficiencies (Pfaffl, 2001). Primer sequences for α-tubulin were 5′-CGA CCA TAA AAG CGC AGT CA-3′ and 5′-CTA CCC AGC ACC ACA GGT CTC T-3′. Primer sequences for *Afrlea1* were 5′-GTG CCG TCT GTG CTC TC-3′ and 5′-CAG GGA GCC TAT GAG GGA CT-3′. Primer sequences for *Afrlea2* were 5′-GAA TGT GCA GCA TCA GCA GT-3′ and 5′-GCT CAG TCA ACA TAT GAC CCA GTG-3′. Values for the fold change in expression are given as means ± SD, and significance was assessed by applying the *t*-test of the mean with Minitab 1998 (Minitab Inc., State Collage, PA).

Bioinformatic analyses

Sequences were compared with the GenBank/NCBI database using BLAST software, and also to the Pfam database (www.sanger.ac.uk/Software/Pfam/search.shtml). The Kyte and Doolittle algorithm (Kyte and Doolittle, '82) was used to construct hydropathy plots (ProtScale program, http://au.expasy.org/tools/protscale.html). A 9-residue moving average was used to compute the hydropathy score. The Biology Workbench 3.2 site (http://workbench.sdsc.edu/) was used for multiple sequence alignments (CLUSTALW) and evaluation of amino acid abundance (AASTATS). Secondary structure predictions were run with the GOR IV, PHDsec, HNN, SSpro, SOPMA, Porter, and Prof programs (http://au.expasy.org/tools/), and values for alpha-helix content were averaged. Subcellular localization of proteins was predicted using: PREDOTAR (www.inra.fr/predotar/), TargetP (www.cbs.dtu.dk/services/TargetP), and Mito Prot (http://ihg.gsf.de/ihg/mitoprot.html). Both proteins were evaluated for regions that could be involved in coiled-coil structures using the

program COILS (www.ch.embnet.org/software/COILS_form.html). Multiple sites for coiled-coil structures were identified for AfrLEA1 (at least 6) and AfrLEA2 (at least 3).

RESULTS AND ANALYSIS

Based on data mining of a cDNA library prepared from *A. franciscana* embryos, coupled with real-time quantitative PCR (rtqPCR), we find that each developmental stage with the capacity for anhydrobiosis (diapause and post-diapause embryos) expresses high levels of two LEA mRNAs, while the desiccation-intolerant larval stage (control) shows expression that is many fold lower (Fig. 1A). This differential expression is consistent with a role for these gene products in survivorship during dehydration.

Deduced protein sequences indicate that AfrLEA1 is composed of 357 amino acids with an apparent molecular mass of 39 kDa, while AfrLEA2 contains 364 amino acids and is 39 kDa. (Table 1). Both proteins are strongly hydrophilic based on hydropathy plots (Fig. 1B). Further, the hydropathy patterns clearly reveal repeating motifs characteristic of plant LEA proteins. Sequence analyses show conserved repeats of 32 amino acids for AfrLEA1 and 14 for AfrLEA2; the tandem repeats become more degenerate toward the carboxy terminus (Fig. 1C, D). The repeating motifs do not match either the historical 11-mer repeat seen for some group 3 LEA proteins (Dure, '93; Cuming, '99), or the 20-mer sometimes seen for group 1 LEA proteins (Esperlund et al., '92; Cuming, '99). However, lack of concordance with the historical repeats is observed even for plant LEA proteins (cf. Grelet et al., 2005).

Comparisons with the GenBank database reveal strong similarities to a phylogenetically broad suite of group 3 LEA proteins for AfrLEA1 (e.g., accession numbers: NP_001024042, *Caenorhabditis elegans*, nematode, E value = $5e^{-15}$; AAA85367, *Picea glauca*, white spruce, $1e^{-08}$; CAA 80491, *Glycine max*, soybean, $1e^{-06}$; YP604937,

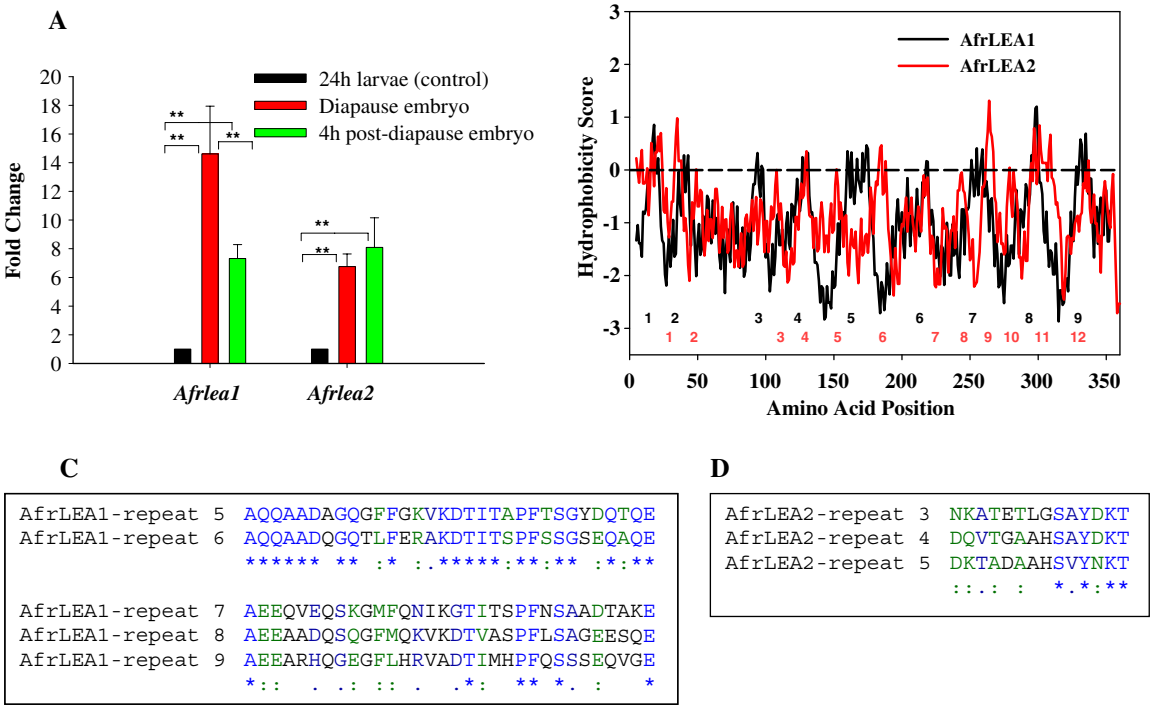

Fig. 1. (**A**) mRNA expression profiles for the LEA genes *Afrlea1* and *Afrlea2* from *Artemia franciscana* embryos. LEA mRNAs are maintained 7–14-fold higher in the two desiccation-tolerant embryonic stages (i.e., diapause and post-diapause) compared to the desiccation-intolerant nauplius larva that served as a control (mean ± SD; $N = 4$–5; $P < 0.005$). The double asterisks indicate that the paired means are statistically different. All ΔCt values (corrected for efficiency) were normalized to the expression of a housekeeping gene (α-tubulin). (**B**) Kyte and Doolittle hydropathy plots for the deduced proteins AfrLEA1 and AfrLEA2 indicate strong hydrophilicity, as shown by values below zero. Amino acid position is plotted on the *x*-axis beginning with the N-terminus. The more evident cyclic repeats are numbered sequentially above the *x*-axis for each protein (color coded). Alignments of tandemly repeated motifs, which correspond to the numbered hydropathy patterns, are provided in (**C**) and (**D**). The repeats become more degenerate as one moves toward the C-terminus.

TABLE 1. Deduced sequences for LEA proteins AfrLEA1 and AfrLEA2 from Artemia franciscana embryos

AfrLEA1						
MAEPEEPPGI	YEKVKSAFVS	APDRAQEAYN	QAYESARSVF	DDAVRSARKM	KNTAAEQAQG	60
AYEGLKESPE	NLQRVTRDIY	HQAQDTGKGA	YETVAGSADD	AYRRAQETAQ	AAQEQSKGFL	120
NRVKDTLTAP	FSSSSDQAKE	TYDRTKDEAQ	YRAQQAADAG	QGFFGKVKDT	ITAPFTSGYD	180
QTQEGYERAR	RSAEEAAQQA	ADQGQTLFER	AKDTITSPFS	SGSEQAQESF	ERAKRAAEEQ	240
VEQSKGMFQN	IKGTITSPFN	SAADTAKEAG	QRAKKQAEEA	ADQSQGFMQK	VKDTVASPFL	300
SAGEESQEAI	ERTKREAEEA	RHQGEGFLHR	VADTIMHPFQ	SSSEQVGEAA	DRIKRGA	357
AfrLEA2						
MPKAAAKGIG	ETVKADADVV	EGMASTGYEK	LKSAFGIASN	KTKDAAENVA	ESARATKDYT	60
VDSAKSAYDK	TVDSTKSAYD	KTTDSAKSVH	DSTADTAKSA	YNKATETLGS	AYDKTKDTAQ	120
STYDQVTGAA	HSAYDKTAEA	TKSAYDKTAD	AAHSVYNKTG	DAGKQAYDST	KEAARSTGKS	180
ISDAAYFTGK	GAERQGDQVK	SELPSYSPSS	SGEKLAQHLV	KSEKEGKKLT	EEALKDRDLS	240
QVPGFRSVKK	AHEPDAKEDI	SAVDFASASP	SQRKVADTEG	VWSSPVDRQE	SRFFSDLAGK	300
IGDMLGGGKI	NAIQTPEEMD	HERLIHKSSQ	SQVAGNVPGR	AKTAWTPEDR	IILHQERFPK	360
ENPE						364

Deinococcus radiodurans, non-spore-forming bacterium, $9e^{-05}$) and for AfrLEA2 (e.g., BAA11017, *Arabidopsis thaliana*, thale cress, $2e^{-14}$; AD59387, *Brassica napus*, rapeseed, $5e^{-15}$; ABA26579, *Phaseolus vulgaris*, bean, $7e^{-13}$; CAF32327, *Pisum sativum*, pea, $3e^{-11}$). Wise (2003) has emphasized that in addition to strong sequence similarities to canonical LEA proteins, upregulated expression profiles that are tightly associated with desiccation, as we report here, are also important for LEA protein identification. In this context, a LEA-like DNA sequence from a chironomid insect larva is present in GeneBank (accession number BAE92616), but expression data are unavailable.

Other features consistent with assignment of AfrLEA1 and AfrLEA2 to group 3 are: high alpha-helix content (77%, AfrLEA1; 59%, AfrLEA2) with significant potential for coiled-coil structures, over-representation of alanine (16.2%, AfrLEA1; 14.3%, AfrLEA2), and the lack of over-representation for glycine or arginine. Both sequences are devoid of cysteine, a feature of many LEA proteins. Finally, when the two *A. franciscana* sequences were compared against the Pfam database, both were assigned to Pfam LEA 4 family, which corresponds to group 3 LEA proteins (cf. Grelet et al., 2005).

DISCUSSION

To our knowledge, this is the first report of two LEA proteins expressed in one animal species. Targeting to different cellular locations might explain the functional significance of two LEA proteins, although bioinformatic analyses with subcellular targeting programs failed to suggest localization to specific organelles such as the mitochondrion. The predictive power of these current software programs is limited, and the complexity of the N-terminal cleavable extensions (termed presequences) involved in targeting is high (Pfanner and Geissier, 2001; Wiedemann et al., 2004).

The expression of LEA proteins is not restricted to plants, having been documented in bacteria, fungi, nematodes (Wise and Tunnacliffe, 2004; Hoekstra, 2005, and references therein), and now a desiccation-tolerant crustacean, *A. franciscana*. This eucoelomate species is the most highly evolved animal for which LEA gene expression has been reported. Among various physiological roles, stabilization of sugar glasses is often suggested for LEA proteins (Hoekstra, 2005), along with protein stabilization via protein–protein interaction (Grelet et al., 2005), ion sequestration (Grelet et al., 2005), and formation of structural networks (Wise and Tunnacliffe, 2004). Such networks have been hypothesized to increase cellular resistance to physical stresses imposed by desiccation (cf. Goyal et al., 2003). It is becoming clear that an ensemble of micromolecules and macromolecules is important for establishing the physical conditions required for cellular stabilization during drying in nature.

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