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Dehydration-induced expression of LEA proteins in an anhydrobiotic chironomid ☆

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Abstract

Late embryogenesis abundant (LEA) proteins are associated with desiccation tolerance in anhydrobiotic organisms. The larvae of an African chironomid, *Polypedilum vanderplanki*, are able to withstand almost complete desiccation during which they enter a state of suspended animation. Here, we developed an EST database from desiccating larvae and isolated three cDNAs encoding proteins (PvLEA1, PvLEA2, and PvLEA3) with highly significant matches to Group 3 LEA proteins. Both mRNA and protein levels of all three examples were increased by dehydration stress imposed by either desiccation or hypersalinity, and one protein, PvLEA2, is likely to be post-translationally processed into smaller molecules. This first description of LEA protein genes in arthropods suggests that this protein family is widespread throughout invertebrate phyla, and that animals, plants, and microorganisms possess similar mechanisms for combating dehydration stress.

Keywords: LEA protein; *Polypedilum vanderplanki*; Anhydrobiosis; Dehydration-inducible gene; Desiccation; Salinity stress; Post-translational processing; Hydrophilic protein; Heat-soluble protein

Late embryogenesis abundant (LEA) proteins are associated with desiccation tolerance in orthodox seeds and resurrection plants, and have been proposed to act as ion scavengers, molecular chaperones, or as cytoskeletal components [1], although molecular mechanisms are not fully elucidated. Recent studies reported that LEA-like proteins are also present in nematodes [2,3] and a rotifer [4]. In addition, LEA homologues were found in genomic information of non-anhydrobiotic nematodes and prokaryotes [5]. These findings suggest that LEA proteins are involved in desiccation tolerance in animals as well in plants, and we therefore hypothesized that an anhydrobiotic chironomid, *Polypedilum vanderplanki*, might also contain LEA-like proteins.

The aquatic larvae of *P. vanderplanki* withstand severe drought in an almost completely dehydrated state during the dry-season in semiarid areas [6,7], termed ''anhydrobiosis'' [8]. In the laboratory, the larvae require 48-h to enter anhydrobiosis successfully, during which expression of various genes is upregulated, trehalose is accumulated, and intra- and extracellular environments are drastically changed [9–11]. In the present study, we thus developed an EST database of desiccating larvae of *P. vanderplanki* and screened drought-inducible genes to identify LEA-like proteins.

Materials and methods

Insects. *Polypedilum vanderplanki* was reared on a milk agar diet under a controlled light condition (13 h light:11 h dark) at 27 °C [9]. To induce expression of anhydrobiosis-related genes, final instar larvae (approxi-

☆ *PvLea1* gene (DDBJ Accession No. AB207255); *PvLea2* gene (DDBJ Accession No. AB207256; *PvLea3* gene (DDBJ Accession No. AB207257); *PvRpl32* gene (DDBJ Accession No. AB244986).

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mately 1 mg wet body mass) were desiccated by a procedure described in a previous report [10].

Cloning of PvLea1, PvLea2 and PvLea3. To establish the EST database of *P. vanderplanki*, cDNA libraries were constructed using total RNAs from the larvae at 0, 12, and 36 h of desiccation process (Hitachi Instruments Service). More than 8000 cDNA clones were sequenced and a database constructed from which three clones for *Lea*-like genes were identified and designated *PvLea1*, *PvLea2*, and *PvLea3*. Full-length cDNA was obtained by 5′- and 3′-RACE using a SMART RACE cDNA Amplification kit (Clontech) with specific primers (Supplementary information). DNA and protein sequences were analyzed with GENETYX-MAC (Genetyx). Protein hydropathy was predicted by Kyte–Doolittle hydropathy plot analysis (http://occawlonline.pearsoned.com/bookbind/pubbooks/bc_mcampbell_genomics_1/medialib/activities/kd/kyte-doolittle.htm). Secondary structure of protein was predicted by "The PSIPRED Protein Structure Prediction Server" (http://bioinf.cs.ucl.ac.uk/psipred/). Motif analysis was performed with Pfam (http://www.sanger.ac.uk/Software/Pfam/).

Heat solubility assay. Recombinant PvLEA1, 2, and 3 proteins containing 6× His-Tag were synthesized with a hybrid baculovirus expression system, extracted with 20 mM potassium phosphate, 10% glycerol, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM PMSF, 10 mM benzamidine, 1 mM DTT (pH 7.5), solubilized by adding Triton X-100 (1% final volume) and purified with Ni^{2+}-affinity chromatography (Superworm system: Katakura Co.). Each extract was dissolved in 50 mM potassium phosphate, 0.3 M NaCl, 10% glycerol, and 0.1% Tween 20 (pH 8.0) in a test tube, heat-treated in boiling water for 15 min, and subsequently centrifuged at 11,000*g* for 15 min to separate into precipitate and supernatant. Each fraction was subjected to Western blot analysis with anti-His antibody (Santa Cruz Biotechnology).

Western blot analysis. Proteins were extracted from desiccating larvae with 20-fold volume of tissue-protein extraction reagent (T-PER, Pierce Biotechnology) containing a protease inhibitor cocktail (Complete: Roche Diagnostics) and 20-μg protein equivalent was subjected to SDS–PAGE and subsequently transferred onto PVDF membrane. The membrane was treated with either anti-AavLEA1 polyclonal antibody [12] or anti-PvLEA2 polyclonal antibody, and then goat anti-Rabbit IgG (H+L) conjugated with horseradish peroxidase (American Qualex). Anti-PvLEA2 was raised against recombinant PvLEA2 and purified by affinity-chromatography using Protein A (Japan Ramb). Immunoreacted proteins were detected by ECL-plus reagent (Amersham Biosciences) and analyzed by LAS-3000 (Fuji Film).

Northern blot analysis. Total RNA was isolated from dehydrating larvae with TRIzol (Invitrogen). Fifteen micrograms of total RNA was run on a 1% agarose-20 mM guanidine isothiocyanate gel and transferred onto Hybond N-Plus membrane (Amersham Biosciences). The probes, full-length *PvLea1*, 2, and 3 cDNA fragments, were synthesized by RT-PCR and labeled with [α-^{32}P]dATP using Strip-Ez labeling kit (Ambion). After hybridization and washing, the membranes were analyzed by BAS 2500 (Fuji Film).

Real-time PCR. cDNA was synthesized from 5 μg of the total RNA using with a Ready-To-Go T-primed First-Strand kit (Amersham Biosciences). Quantitative PCR analyses were performed on a LightCycler 2.0 system (Roche diagnostics). Primer sets are listed in Supplementary information. PCRs run under a thermal cycling condition: 95 °C for 10 min and then 45 cycles of 95 °C, 60 °C, and 72 °C every 10 s. Data were analyzed with the LightCycler software 4.0 (Roche diagnostics). Results were corrected by a value of an internal control, *P. vanderplanki* Ribosomal protein L32 (*PvRpl32*; Accession No. AB244986), whose mRNA remained constant during desiccation (data not shown).

Statistical analysis. Data were analyzed by two-tailed multiple *t*-test with Bonferroni's correction following two-way ANOVA (Prism version 4; GraphPad Software).

Results

Cloning of PvLea1, PvLea2 and PvLea3 genes

We first attempted to isolate genes encoding LEA-like protein from *P. vanderplanki*. LEA proteins are subclassed into several groups [13], and since all animal LEA proteins described to date belong to Group 3 [2,3, McGee and Tunnacliffe, unpublished data], we expected any chironomid LEA-like proteins to be of the same group. Group 3 LEA proteins are defined by repeated 11-mer motifs [5], but the consensus 11-mer sequence is not very highly conserved [13]. For such loosely conserved sequences, conventional methods of cloning such as colony hybridization using cDNA library and RT-PCR using degenerated primers are unlikely to be successful. Therefore, we decided instead to search for target genes in a novel EST database constructed from cDNA libraries of desiccating chironomid larvae. After clustering of identical clones, we picked up three clusters annotated as LEA-like proteins (Supplementary data, Table S1). No or few clones of the *Lea*-like gene clusters were apparently expressed before desiccation challenge (DP 0), but clone numbers markedly increased after 12 h of desiccation (DP 12), and high numbers of clones were maintained subsequently (DP 36). Using 5′- and 3′-RACE, we cloned three independent genes designated *PvLea1* (DDBJ Accession No. AB207255), *PvLea2* (AB207256), and *PvLea3* (AB207257). Genomic PCR showed that *PvLea1*, *PvLea2*, and *PvLea3* genes consist of at least 4, 1, and 4 exons, respectively (data not shown). Each cDNA sequence has single open-reading frame.

This is the first cloning of *Lea*-like genes from arthropods including insects. By blast search (http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi?organism=insects), we found no significant orthologue of *PvLea* genes in the genome databases of other insects, i.e., *Drosophila melanogaster* (fruit fly), *Anopheles gambiae* (mosquito), *Bombyx mori* (silkworm), *Apis mellifera* (honey bee), and *Tribolium castaneum* (flour beetle).

Hydrophilic character of PvLEA1, PvLEA2, and PvLEA3 protein

Predicted molecular weights of PvLEA1, PvLEA2, and PvLEA3 were 83.2, 20.6, and 54.6 kDa. Deduced amino acid sequences of cloned PvLEA proteins contain multiple "LEA_4" motifs (Pfam Accession No. PF02987: http://www.sanger.ac.uk/cgi-bin/Pfam/getacc?PF02987), with very high similarity (Fig. 1A). The LEA_4 motif contains over 4 repetitions of the 11-mer sequence characteristic of the Group 3 LEA proteins [13]. In common with other Group 3 LEA proteins, secondary structure prediction programs (PSIPRED) suggest that PvLEA proteins predominantly form amphipathic α-helices throughout the "LEA_4" motif regions (Fig. 1A). These results are consistent with categorization of PvLEA1, PvLEA2, and PvLEA3 as Group 3 LEA proteins, similar to AavLEA1, the LEA protein described in the anhydrobiotic nematode *Aphelenchus avenae* [2,12].

In silico analysis such as Kyte–Doolittle hydropathy plot predicted that all three PvLEA proteins are predominantly hydrophilic, with few hydrophobic residues (e.g., the N-terminus of PvLEA1 protein and both ends of PvLEA3) (Fig. 1B). It is likely that their extreme hydrophilicity

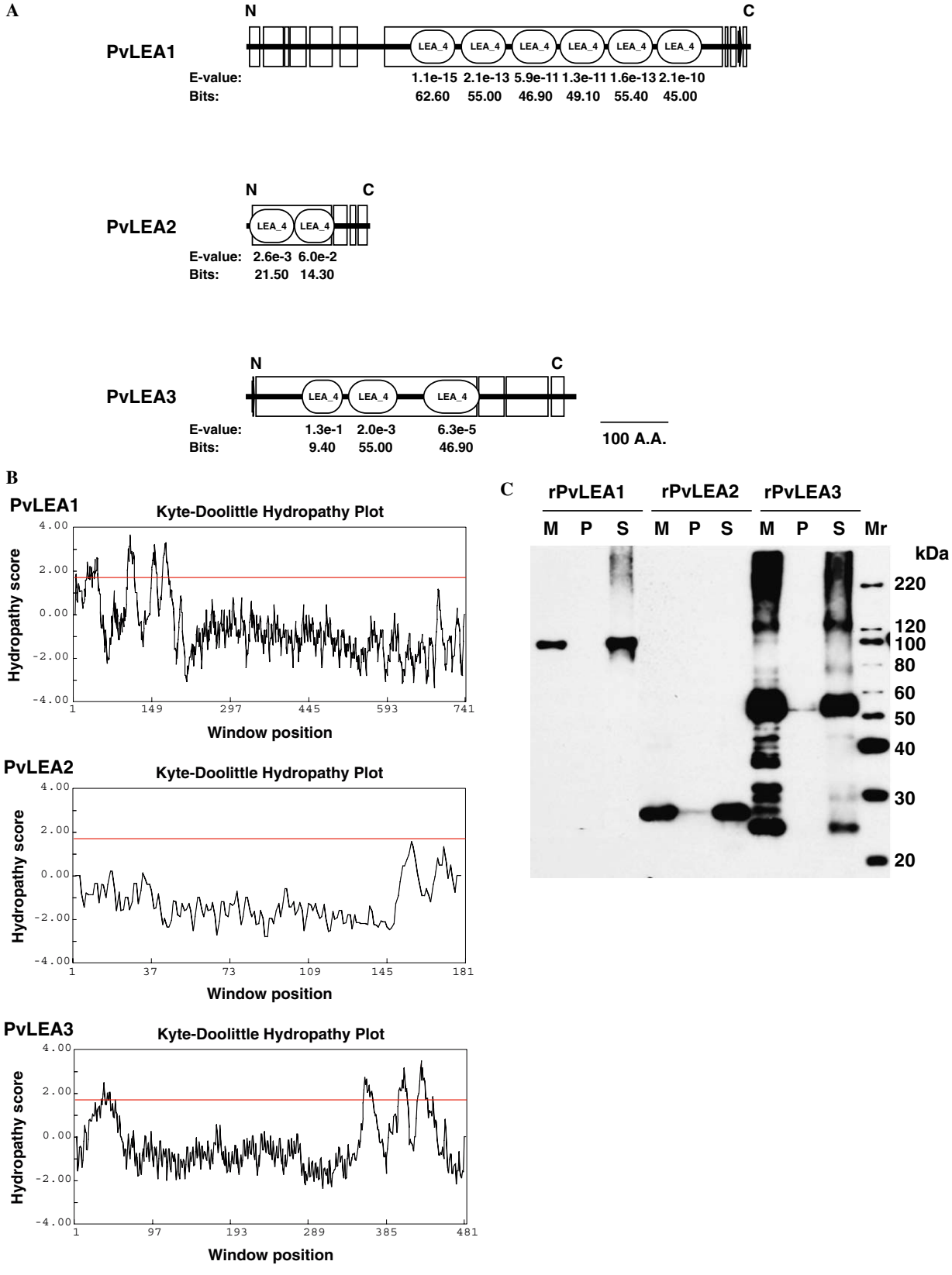

Fig. 1. Hydrophilic character of PvLEA1, PvLEA2, and PvLEA3. (A) Schematic illustrations for predicted structures of PvLEA proteins. Oval figures indicate "LEA_4" motifs. Higher bits and lower E-value scores suggest higher similarity to the domain. Rectangles indicate regions with the potential to form amphipathic α-helices. The scale bar indicates a length of 100 amino acid residues. (B) Kyte-Doolittle hydropathy plots for PvLEA proteins. A value for parameter of window size was set at 9. Straight line indicates threshold (value = 1.8) for hydrophobic region. (C) Heat solubility of PvLEA proteins. Recombinant PvLEA proteins containing 6× His-Tag were heat-treated and subjected to Western blot analysis using anti-His antibody. M, purified rPvLEA without heat-treatment; P, precipitated proteins after boiling; S, soluble proteins after boiling.

inhibits folding in aqueous solution and that they are at least partially natively unfolded [12]. As is generally known, hydrophilic LEA proteins do not aggregate and precipitate even after boiling [1]. Accordingly, recombinant PvLEA proteins (rPvLEA1, 2, and 3) remained in the soluble fraction after heat treatment (Fig. 1C, lane S), indicating that PvLEA proteins have similar physico-chemical properties to other LEA proteins.

Dehydration-induced expression of PvLea1, PvLea2, and PvLea 3

Next, we examined temporal changes of *PvLea* expression during desiccation. Three *PvLea* genes showed a similar expression pattern in Northern blot analysis, although expression levels were somewhat different (Fig. 2A). *PvLea* mRNAs were slightly expressed even in an unstressed larva (0 h of desiccation). Vigorous expression was induced by a desiccation stress and continued until drying was complete (48 h). Real-time PCR confirmed the desiccation-inducible expression profiles of *PvLea* mRNAs (Fig. 2C–E). These results indicate that *PvLea* genes are turned on soon after *P. vanderplanki* larvae are removed from water.

We have previously demonstrated that salinity stress induces an anhydrobiosis-like physiological change in *P. vanderplanki*: larvae submerged in 1% NaCl solution start to accumulate trehalose [10], in the same way as larvae removed from water. Expression of *PvLea* genes was also induced by NaCl stress (Fig. 2B–E), indicating that hypersalinity can mimic desiccation in terms of gene

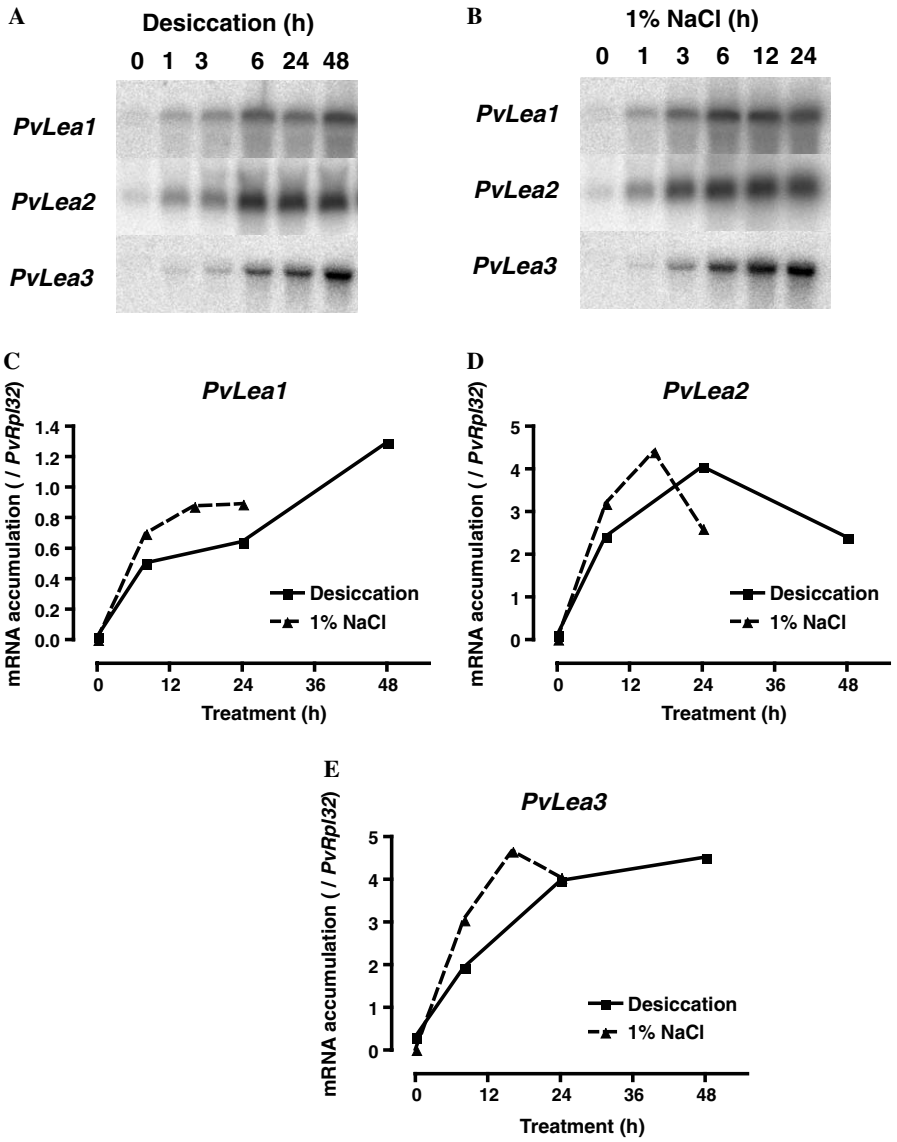

Fig. 2. *PvLea1*, *PvLea2*, and *PvLea3* expression in *P. vanderplanki* larvae experiencing water stress. (A,B) Northern blot analysis of *PvLea1*, *PvLea2* and *PvLea3* mRNA isolated from desiccating larvae (A) and from salinity stressed larvae (B). The number on each lane indicates hours after set up of treatments. (C–E) Temporal changes of *PvLea1* (C), *PvLea2* (D), and *PvLea3* (E) gene expression level analyzed by real-time PCR. Expression of each *PvLea* gene was corrected using expression of *PvRpl32* as a reference.

transcription. This finding supports a hypothesis that desiccation stress and salinity stress activate a common signal transduction pathway in *P. vanderplanki* [10].

Dehydration-induced processing of PvLEA2 protein

Expression of PvLEA proteins during dehydration was examined by Western blotting using anti-AavLEA1 antibody and anti-PvLEA2 antibody. Anti-AavLEA1 antibody recognized several desiccation-inducible proteins including putative full-length PvLEA1 (band a) and PvLEA3 (band c), but a signal corresponding to PvLEA2 was absent (Fig. 3A). On the other hand, anti-PvLEA2 antibody failed to detect proteins of relatively higher molecular weight corresponding to the bands a–f in Fig. 3A, but recognized two desiccation-inducible proteins whose molecular weights are estimated at 19.7 and 14.7 kDa (Fig. 3B, bands h and j). They were absent, or at very low levels, in unstressed larvae, but were also induced by salinity stress (1% NaCl).

The 19.7 kDa protein is probably identical to full-length PvLEA2, whose predicted molecular weight is 20.6 kDa. It was present in desiccating larvae at 8 and 24 h of the desiccation process, but was absent in desiccated larvae at 48 h (Fig. 3B). On the other hand, the protein of 14.7 kDa seemed to be present in much greater quantities than the putative full-length PvLEA2. Its concentration increased during desiccation, and a relatively large quantity is present in desiccated larvae (Fig. 3B). Full-length PvLEA2 might be processed into 14.7 kDa protein in a desiccating *P. vanderplanki* larva, as a similar phenomenon has been reported for AavLEA1 [14,15]. In a similar way, PvLEA1 and PvLEA 3 might give rise to smaller proteins recognized with anti-AavLEA1 antibody (e.g., Fig. 3A, bands b and d–g).

Anti-PvLEA2 antibody detected an additional protein of 16.0 kDa (Fig. 3B, band b), which was present only when larvae were almost completely desiccated whose identity is unexplained. We believe that it was not artificially generated when proteins were extracted from almost completely desiccated material, because other proteins were not degraded (e.g., see Fig. 3A). If our hypothesis that PvLEA2 is processed to the smaller 14.7 kDa polypeptide is correct, however, the 16.0 kDa protein could represent an intermediate, partially processed form. Alternatively, either or both smaller proteins could be unidentified LEA-like proteins.

Discussion

In the present study, we describe the first examples of *Lea*-like genes from an arthropod. An anhydrobiotic chironomid, *P. vanderplanki*, has at least three LEA proteins, which are upregulated by both desiccation and hypersaline stress. PvLEA proteins are designated Group 3 LEA proteins, like previously described proteins from nematodes. AavLEA1 is processed into smaller polypeptides that retain function as protein anti-aggregants in desiccating nematodes. At least one LEA protein from *P. vanderplanki*, PvLEA2, probably also undergoes post-translational processing during dehydration. Since this processing phenomenon has never been reported with plant LEA proteins as far as we are aware, this might indicate a difference in function between plant and animal Group 3 LEA proteins.

When the *lea-1* gene from the model nematode *Caenorhabditis elegans* was silenced by RNA interference, a marked reduction in desiccation resistance in dauer larvae was found [3]. We thus assume that PvLEA proteins contribute greatly to the protection from dehydration stress in anhydrobiosis of *P. vanderplanki*. The hydrophilic

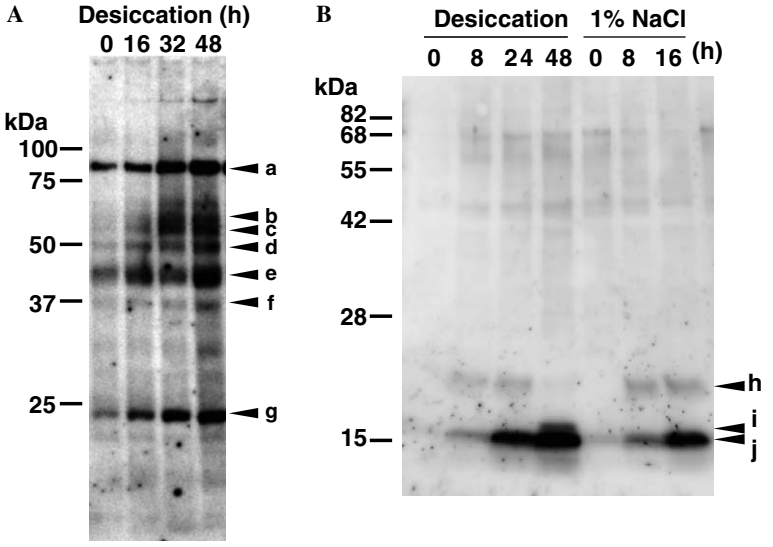

Fig. 3. Expression of PvLEA proteins in *P. vanderplanki* larvae experiencing water stress. (A,B) Western blot analysis of proteins extracted from desiccating *P. vanderplanki* larvae, using anti-AavLEA1 antibody (A) and anti-PvLEA2 antibody (B). The number on each lane indicates hours after set up of desiccation. At 48 h, the larvae were almost completely desiccated. Molecular weights of the proteins indicated by a–j were estimated at approximately 83.1, 57.2, 53.2, 48.6, 42.6, 37.7, 23.8, 19.7, 16.0, and 14.7 kDa, respectively. The positions of molecular weight standards are shown.

nature of LEA proteins, which is thought to be responsible for their resistance to aggregation, could be important for their beneficial role in desiccation tolerance, for example, as a molecular shield. Processing into smaller polypeptides may increase specific activity of the LEA protein and increase the efficiency of protection against dehydration stress [15].

In addition, a Group 3 LEA protein from *Typha latifolia* pollen has been demonstrated to stabilize sugar glasses *in vitro* [16]. Trehalose glass formation is essential for long-term stability of anhydrobiotic status in *P. vanderplanki* (Sakurai and Furuki, personal communications), to which PvLEA proteins may contribute. To confirm this, further studies on the physico-chemical interactions between PvLEA and trehalose will be conducted.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.07.003.

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