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Biochemical and structural characterization of an endoplasmic reticulum-localized late embryogenesis abundant (LEA) protein from the liverwort *Marchantia polymorpha*

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ABSTRACT

Late embryogenesis abundant (LEA) proteins, which accumulate to high levels in seeds during late maturation, are associated with desiccation tolerance. A member of the LEA protein family was found in cultured cells of the liverwort *Marchantia polymorpha*; preculture treatment of these cells with 0.5 M sucrose medium led to their acquisition of desiccation tolerance. We characterized this preculture-induced LEA protein, designated as MpLEA1. MpLEA1 is predominantly hydrophilic with a few hydrophobic residues that may represent its putative signal peptide. The protein also contains a putative endoplasmic reticulum (ER) retention sequence, HEEL, at the C-terminus. Microscopic observations indicated that GFP-fused MpLEA1 was mainly localized in the ER. The recombinant protein MpLEA1 is intrinsically disordered in solution. On drying, MpLEA1 shifted predominantly toward α-helices from random coils. Such changes in conformation are a typical feature of the group 3 LEA proteins. Recombinant MpLEA1 prevented the aggregation of α-casein during desiccation–rehydration events, suggesting that MpLEA1 exerts anti-aggregation activity against desiccation-sensitive proteins by functioning as a "molecular shield". Moreover, the anti-aggregation activity of MpLEA1 was ten times greater than that of BSA or insect LEA proteins, which are known to prevent aggregation on drying. Here, we show that an ER-localized LEA protein, MpLEA1, possesses biochemical and structural features specific to group 3 LEA proteins.

1. Introduction

Liverworts have been identified as the earliest divergent clade of land plants and are considered the first organisms with vegetative desiccation tolerance among terrestrial plants [1]. Accordingly, investigation of the mechanisms that underpin desiccation tolerance in liverworts could contribute to our understanding of how plants acquired desiccation tolerance during the process of foraying onto land. We previously reported that suspension-cultured cells of the liverwort *Marchantia polymorpha* were able to acquire a high level of desiccation tolerance [2]. To induce this desiccation tolerance, the cells were cultured in a preculture medium containing 0.5 M sucrose for 4 days. This preculture treatment of the cultured cells caused accumulation of endogenous proteins, which were induced by dehydration under a high osmolality condition. The accumulation of these preculture-induced proteins was crucial to the acquisition of desiccation tolerance in the cultured cells [3], and one of the preculture-induced proteins, with molecular mass of 35 kDa, appeared to be a group 3 late embryogenesis abundant (LEA) protein [2].

LEA proteins were first reported in the early 1980s and are known to accumulate in maturing seeds [4]. Their accumulation is not only related to the development of desiccation tolerance in orthodox seeds (desiccation-tolerant seeds), but it is also induced upon water-related stress in plant vegetative tissues and other anhydrobiotic organisms [5–9]. LEA proteins are classified into three main groups according to the characteristics of their amino acid sequences [10,11]. Among them, the group 3 LEA proteins are found in many desiccation-tolerant organisms, including plants and animals.

Abbreviations: CD, circular dichroism; CHO, Chinese hamster ovary; ER, endoplasmic reticulum; FT-IR, Fourier transform-infrared; IPTG, isopropyl β-D-1-thio-galactopyranoside; LEA, late embryogenesis abundant.

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Recent studies on the LEA proteins have focused on their biochemical and biophysical function in desiccation tolerance [9,12–14]. Most LEA proteins are highly hydrophilic and soluble upon boiling due to their hydrophilicity [10]. Secondary structure analysis using circular dichroism (CD) or Fourier transform infrared (FT-IR) spectroscopy has shown that these proteins are intrinsically disordered in solution and structured when dehydrated [9,14–16]; several mechanisms, including "molecular shields", "ion scavengers" and "cytoskeletal filaments", have been proposed to be involved in the protection of cellular components during drying [12,17,18].

To date, structural and biochemical analyses have primarily been conducted on LEA proteins localized in the cytoplasm, chloroplasts, and mitochondria, whereas there have been few reports about the endoplasmic reticulum (ER)-localized LEA proteins [11]. Hence the role of LEA proteins at the ER remains unclear. Under hydrated (normal) conditions, the ER plays many pivotal roles in maintaining the functions of proteins, such as glycosylation, disulfide bond formation, protein transport to the Golgi apparatus, and the facilitation of protein folding. Do LEA proteins contribute to sustaining the functional integrity of the ER under desiccating conditions, and if so, how? To answer this question, we analyzed a Marchantia LEA protein, MpLEA1, which was predicted to localize in the ER on the basis of its deduced amino acid sequence. In this study, we describe the biochemical and structural characteristics of this novel LEA protein.

2. Materials and methods

2.1. Cloning of the cDNA encoding MpLEA1

The cDNA prepared from total RNA of *M. polymorpha* as described previously [19] was used as a template for PCR with *MpLea1*-specific primers, 5′-CGTTTCCAGACTCTTACAGG-3′ and 5′-CTGACAGCTACCGTAGTCAT-3′, designed on the basis of the EST database of *M. polymorpha*. The amplified PCR fragment was cloned into the pGEM-T Easy vector (Promega, Madison, WI). The *MpLea1* (GenBank ID: AB616673) cDNA was verified by sequencing. The cDNA and deduced amino acid sequences were analyzed with GENETYX-MAC software (Genetyx, Tokyo, Japan). The Blastp search was performed at the website of the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The Kyte and Doolittle hydropathy plot was computed with a 9-residue average. Bioinformatic analysis of the protein was performed with InterProScan 5 [20], TargetP 1.1 [21,22], and PROSITE [23]. The sequence logo for the 11-mer motif in MpLEA1 was designed by using the web-based application WebLogo 3.4.

2.2. Subcellular localization of MpLEA1–GFP protein in cultured cells

A GFP–*MpLea*1 expression vector, pcDNA3-GFP–*MpLea1*, was constructed as follows: the region encoding the putative signal peptide of MpLEA1 was isolated by PCR using the pGEM-T construct containing *MpLea1* as a template with the T7 forward primer and the antisense primer 5′-CATGCCATGGCGCATCTGACCACAAGAGCAGTGG-3′, and then digested with *Eco*RI and *Nco*I. The deletion construct *MpLea1*-d, which lacked the putative signal peptide in its N-terminus, was amplified by using the sense primer 5′-GTGTACAAGGAGGAGACGATCGGCCAAAAAGT-3′, and the antisense primer 5′-ATAAGAATGCGGCCGCTTACAACTCCTCGTGAGAAGCTTCC-3′, and was then digested with *Bsr*GI and *Not*I. The amplified fragment was inserted into the CaMV35S-sGFP(S65T)-NOS3′ vector [24]. The sGFP(S65T) fused with *MpLea1*-d fragment was digested with *Nco*I and *Not*I. The fragments for the corresponding signal peptide and GFP–*MpLea1*-d were inserted between the *Eco*RI and *Not*I sites of the mammalian expression vector pcDNA3 (Life Technologies, Carlsbad, CA). For the sGFP(S65T) protein, the CaMV35S-sGFP(S65T)-NOS3′ vector was digested with *BamH*I and *EcoICR*I. The small fragment was ligated into the pcDNA3 vector at the *BamH*I and *Eco*RV sites.

For transient expression, each plasmid was transfected into CHO (Chinese hamster ovary)-K1 cells by using the NEPA21 Super Electroporator (NepaGene Co. Ltd., Chiba, Japan). Heterologous expression was allowed to proceed for 24 h. Cells expressing GFP–MpLEA1 and GFP were stained with ER-Tracker™ Red (Life Technologies) and colocalization was assessed by use of a laser scanning microscopy (LSM 700; Carl Zeiss, Jena, Germany).

2.3. Expression and purification of recombinant MpLEA1 protein

MpLea1-d lacking the signal peptide was inserted between the *Nde* I and *BamH* I sites of the pET14b T7 expression vector (Novagen, Merck KGaA, Darmstadt, Germany). The plasmid pET-*MpLea1*-d was introduced into *Escherichia coli* BL21 (DE3) cells, and the expression of the recombinant protein tagged with histidine residues was induced for 3 h by the addition of 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside). Induced *E. coli* cells were heated for 15 min at 93 °C, cooled for 10 min on ice, and centrifuged at 10,000*g* for 10 min at 4 °C. Recombinant MpLEA1 was purified from the resultant supernatant by using Ni^{2+}-affinity chromatography according to the manufacturer's instructions (His·Bind® Kit; Novagen). Eluted recombinant MpLEA1 protein was desalted with water, and lyophilized. Protein purification was monitored by use of SDS–PAGE on a 12% gel followed by CBB staining.

2.4. Sample preparation for FT-IR spectroscopic measurements

Pure recombinant MpLEA1 protein was dissolved in D_2O at 10 mg mL^{-1} and then lyophilized two times for deuterium exchange of the protein. Dry samples were prepared by drying 5-μL droplets of the D_2O solution with the H–D exchanged MpLEA1 at 10 mg mL^{-1} on a Teflon plate in a desiccator (relative humidity at ca. 5%) for at least 24 h.

2.5. FT-IR spectroscopic measurements and analysis

The dry protein films were sandwiched between two KBr plates (Jasco, Tokyo, Japan). Protein samples in D_2O were poured between two circular (φ 20 × 2 mm) CaF_2 plates (Jasco) with a Teflon spacer (ca. 50-μm thick). IR spectra in the range 4000–650 cm^{-1} were recorded at room temperature, with a spectral resolution of 4 cm^{-1} and 128 scans, on an FT/IR-6200 spectrometer (Jasco) equipped with a liquid nitrogen-cooled mercury/cadmium/telluride detector and an infrared microscope (IMV-4000; Jasco). For the secondary structural analysis of MpLEA1, we focused on the spectral region between 1750 and 1550 cm^{-1}, which contained the amide-I absorption bands due primarily to stretch vibration of the carbonyl groups in the peptide bonds on the protein backbones [25]. Secondary structural components were derived from the second derivative spectra of the amide-I band, for which Savitzky–Golay function [26] was used, and incorporated into the software for FT-IR spectral analysis supplied by Jasco.

2.6. Aggregation assay

We chose α-casein as an example of an aggregation-prone protein on drying. Aggregation of α-casein from bovine milk (Sigma–Aldrich, St. Louis, MO) was monitored in a spectrophotometer (SPECTRAMAX PLUS; Molecular Devices, Sunnyvale, CA) by reading absorbance A at 340 nm and 280 nm. Drying was performed in a centrifugal concentrator (VC-960; TAITEC, Saitama, Japan)

equipped with a high vacuum pump. Two hundred-microliters of α-casein (2 mg/ml) was dried in the absence or presence of MpLEA1 for 2.5 h and then the dried samples were rehydrated in 200 μL of water. The rehydrated samples were then re-dried and rehydrated in 200 μL of water again. Statistical significance was determined by use of one-way ANOVA and Tukey's post hoc test by using Prism 6 (GraphPad Software, La Jolla, CA).

3. Results

3.1. Sequence analysis of MpLEA1

MpLea1 encodes a polypeptide of 319 amino acid residues with a predicted molecular mass of 33,255 Da and a pI value of 5.01. A Blastp search indicated that the deduced amino acid sequence of the cloned *MpLea1* showed significant homology to group 3 LEA proteins [e.g., *E*-values to DC-8-like embryonic proteins of *Solanum tuberosum* (GenBank ID: XP_006366996.1 and XP_006340631.1): $3e^{-10}$ and $2e^{-8}$; late embryogenesis abundant protein D-29 of *Vitis vinifera* (GenBank ID: XP_002266501.1): $1e^{-8}$]. MpLEA1 (positions 31–311) is classified into the late embryogenesis abundant (plants) LEA-related family (PTHR 23241) (Fig. 1A). This protein consists of 7 repeating units of an 11-mer amino acid motif and the consensus sequence based on the frequency of occurrence for each residue is AKDTAAKKVDE (Fig. 1B), a characteristic of the group 3 LEA proteins. TargetP predicted that 25 amino acid residues at the N-terminus of MpLEA1 could be the signal peptide. In fact, the protein sequence analysis revealed that the mature form of MpLEA1 was truncated the region for the putative signal peptide [2]. The PROSITE program showed that MpLEA1 has an ER targeting sequence, His-Glu-Glu-Leu (HEEL), at the C-terminus (Fig. 1A). According to the Kyte–Doolittle hydropathy plot, MpLEA1 could be predominantly hydrophilic with a few hydrophobic residues at the N-terminus (Fig. 1C). The high hydrophilicity of MpLEA1 most likely contributed to its high solubility under heating. Indeed, the endogenous [2] and recombinant (Supplemental Fig. 1) MpLEA1 proteins remained in the soluble fraction even after heating at 93 °C for 15 min.

3.2. Subcellular localization of MpLEA1–GFP fusion protein in cultured cells

Intracellular MpLEA1 protein localization was investigated in cultured mammalian cells because mechanisms underlying the protein trafficking are highly conserved in eukaryotes [27]. In the cells expressing GFP–MpLEA1, overlap of the red and green fluorescence signals was observed, indicating colocalization with ER-Tracker Red (Fig. 2A). As a positive control, we used an ER-GFP that contained the *Arabidopsis thaliana* chitosan signal peptide at the N-terminus and the retention signal KDEL at the C-terminus [28]. We confirmed that this plant ER marker, ER-GFP, also colocalized with the red fluorescence signal for ER-Tracker in mammalian cells (data not shown). In contrast, GFP alone was distributed both in the cytoplasm and in the nucleus (Fig. 2B). These results show that MpLEA1 is indeed an ER-localized protein.

3.3. Secondary structure of MpLEA1

To investigate the structural and functional properties of MpLEA1, the recombinant protein without the N-terminal signal peptide was purified and lyophilized (Supplemental Fig. 1). Secondary structures of the recombinant protein in solution and after drying were analyzed with FT-IR (Fig. 3). The IR spectrum of the purified protein in solution was initially recorded. The protein was dissolved in D_2O to avoid interference from the H–O–H scissoring vibration with the amide-I band between 1700 and 1600 cm^{-1}. For the D_2O solution of MpLEA1, a broad band at 1645 cm^{-1} was visible in the amide-I region of the spectrum. Its second-derivative spectrum was calculated to detect possible secondary structural components appearing in the amide-I band more precisely. One major band with maximum absorption at 1645 cm^{-1} was assigned to random coil structures [25], indicating that the protein in solution was highly disordered. In the dry state, on the other hand, the band at 1655 cm^{-1} assigned to α-helical structures [25] was predominant in the second-derivative spectrum of the amide-I region. This finding indicates that in a dry state the protein adopts a conformation of predominantly α-helical structures.

3.4. MpLEA1 prevents α-casein aggregation during dehydration-rehydration events

The molecular shield activity, one of the mechanisms of LEA protein function, limits desiccation-induced aggregation [29]. To verify a role for MpLEA1 as a molecular shield, we measured the ability of MpLEA1 to reduce α-casein aggregation during desiccation–rehydration events by using a turbidity measurement. After each desiccation–rehydration event, irreversible aggregates were

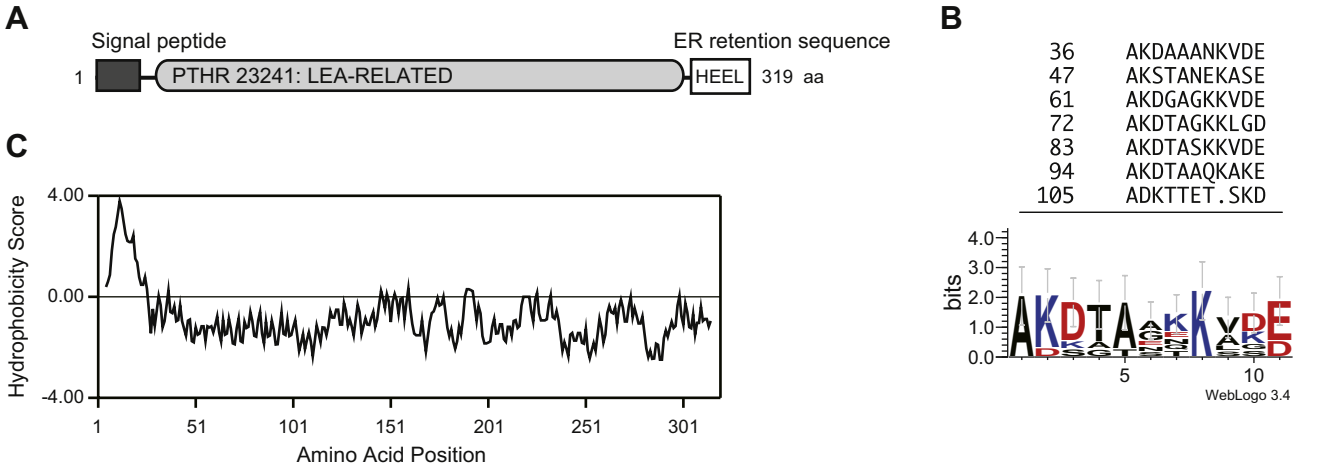


Fig. 1. The features of MpLEA1. (A) Schematic illustration of the predicted structure of MpLEA1. Oval figure indicates for PTHR 23241. The 25-amino acid N-terminal signal sequence is shown in dark gray. MpLEA1 has a putative ER retention signal HEEL at the C-terminus. (B) Alignment of the 11-mer motifs optimized by introducing a gap indicated as a dot. Numbers refer to amino acid positions. A sequence logo illustrates the 11-mer motif in MpLEA1. (C) Kyte and Doolittle hydropathy plot for MpLEA1 indicates strong hydrophilicity (shown by values below zero). The more hydrophobic N-terminal 25-amino acid region is indicative of a signal sequence.

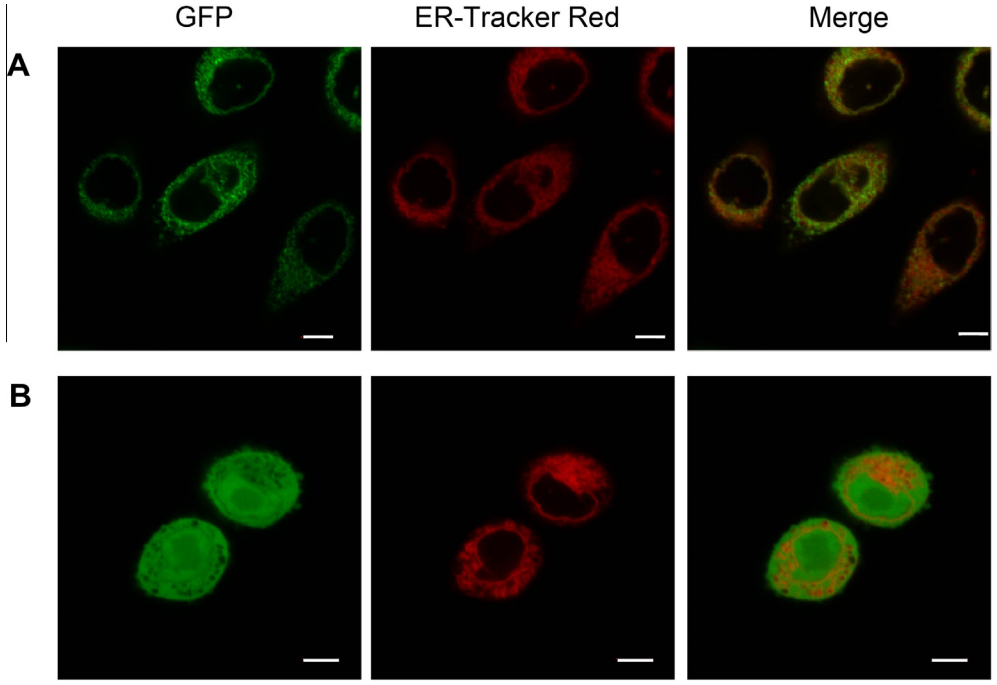


Fig. 2. Transient expression of MpLEA1 protein located in the ER. GFP fluorescence images of CHO cells transiently transfected with a vector expressing (A) a GFP-fused *MpLea1* gene and (B) a GFP gene. Green, GFP; red, ER-Tracker Red. Bars = 5 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

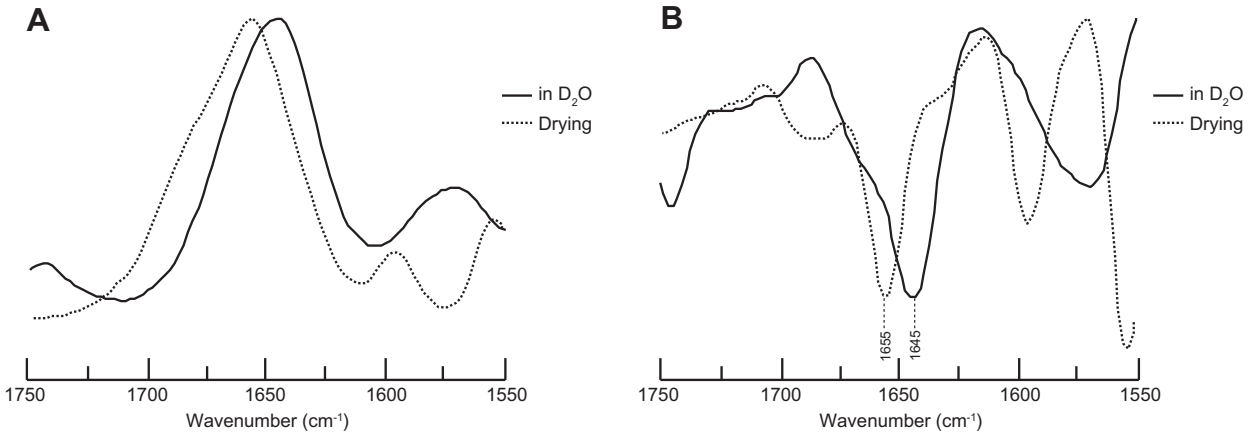


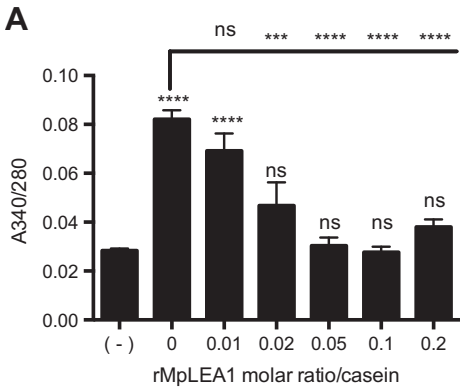
Fig. 3. FT-IR spectroscopy of MpLEA1 in D_2O and after drying. (A) FT-IR absorption spectrum in the amide-I region of the protein. (B) Second-derivative spectrum. The protein in D_2O was highly disordered, being largely in a random coil conformation (band at 1645 cm^{-1}). The protein in its dry state exhibited a largely α-helical conformation (band at 1655 cm^{-1}).

formed in the α-casein solution and the turbidity in the solution increased 2.9-fold (Fig. 4A). MpLEA1 reduced this increase in turbidity in the α-casein solution after desiccation. When MpLEA1 was added at molar ratios to α-casein of 0.02 or more, the turbidity of the solutions after drying was the same as that of the solutions before drying (Fig. 4A). When viewed from the perspective of usage, MpLEA1 was ten times more effective than BSA and PvLEA4 proteins, which are known to prevent aggregation on drying (Fig. 4B). These results indicate that MpLEA1 prevented the aggregation of α-casein during desiccation–rehydration events by functioning as a molecular shield.

4. Discussion

In this study, we characterized a novel LEA protein from liverwort and identify it as a group 3 LEA protein in Marchantiales. This LEA protein, MpLEA1, was found as a preculture-induced, heat-soluble protein in desiccation-tolerant *M. polymorpha* cultured cells [2]. The amino acid sequence of MpLEA1 showed similarity to those of the DC-8 like protein of *S. tuberosum* and D-29 of *V. vinifera*, which are also thought to be members of the group 3 LEA proteins. MpLEA1 localized to the ER. It has a signal sequence at its N-terminus and an ER retention signal, HEEL, at its C-terminus. Among eukaryotes, including mammals, the C-terminal sequence HEEL has been shown to function as an ER retention signal [30].

FT-IR spectroscopic analysis showed that the conformation of the recombinant MpLEA1 protein predominantly shifted to α-helices from random coils upon drying (Fig. 3). Such a change in conformation is a characteristic of group 3 LEA proteins, being previously observed in anhydrobiotic nematode AavLEA1 and pea LEAM [14,15]. Bioinformatics studies predicted that the 11-mer sequences form α-helices as amphiphilic filaments upon desiccation, which then form dimers via interactions among each



B

	Minimum molar ratio/ Casein	Minimum weight ratio/ Casein
MpLEA1	0.02	0.035
BSA[(a)]	0.2	0.7
PvLEA4[(a)]	0.2	0.16

[(a)] These data were modified from Ref. [9].

Fig. 4. Anti-aggregation activity of MpLEA1 under desiccation and rehydration cycles. The turbidity in the mixtures, which were concentrated by centrifugation and then rehydrated, was measured in a spectrophotometer as the ratio of absorbance at 340 and 280 nm. MpLEA1 was added to the α-casein solutions at the indicated molar ratios. (-) indicates samples before drying. This assay was performed in at least triplicate and the standard deviation is shown.

hydrophobic region [17]. Indeed, AavLEA1 has been shown to form dimers and oligomers on drying [15]. Such previous findings led to the hypothesis that group 3 LEA proteins confer desiccation tolerance on drying cells by increasing mechanical strength via filamentous structures of coiled coil oligomers [18]. Thus far, CD and FT-IR spectroscopic analyses have revealed that group 3 LEA proteins, localized in the cytoplasm, mitochondria, or chloroplasts, are predominantly unstructured in solution and become mainly α-helical upon desiccation [14–16]. Secondary structures of the bdelloid rotifer's two ER-localized LEA proteins have also been examined by using CD spectroscopy [31]. On drying, one of these proteins adopted the structure of a classical group 3 LEA protein, but the other formed a structure that was representative of both the hydrated and dry states, which is unique for a group 3 LEA protein [31]. In our study, the ER-localized LEA protein MpLEA1 changed its secondary structure upon dehydration, just as most other group 3 LEA proteins have been shown to do. The accumulation of such findings compels us to argue that regardless of their localization, the majority of group 3 LEA proteins likely share a common biophysical feature with respect to conformational changes in their secondary structures.

Based on the structural features of group 3 LEA proteins, other mechanisms for desiccation tolerance have been proposed, such as prevention of protein aggregation via interaction with charged amino acid residues, as molecular shields [12,17,18]. In the current study, MpLEA1, the ER-localized LEA protein from liverwort, was shown to reduce the formation of α-casein aggregates during desiccation–rehydration events by functioning like a molecular shield (Fig. 4). Under normal, hydrated conditions, the ER plays many pivotal roles in maintaining the functions of proteins. Accumulation of unfolded proteins in the ER causes the unfolded protein response (UPR) [32,33]. Several ER chaperon proteins, including Hsp70 family member BiP, conduct correct folding of newly synthesized proteins by interacting with unfolded proteins. Overexpression of BiP in tobacco protoplasts alleviates ER stress [34]. Likewise, MpLEA1 would react on alleviation of the ER stress by preventing aggregation of unfolded protein under drying conditions. It is, therefore, possible that MpLEA1 contributes to cell protection against desiccation damage.

Although there have been many group 3 LEA protein functional analyses, the precise role of these proteins remains to be clarified. The specific features of LEA protein conformation suggest that these proteins could have a wide variety of functions in stress tolerance as moonlighting proteins. Therefore, we may find a new function of LEA proteins in stress tolerance at the ER, which has not previously been considered a site involved in the mechanisms of desiccation tolerance. Further studies are needed to clarify the role of MpLEA1 in desiccation tolerance.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.10.130.

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