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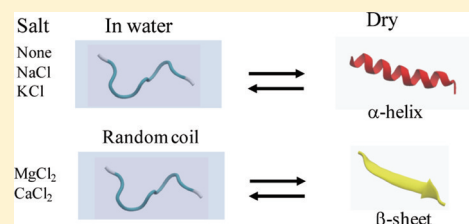
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## Supporting Information

**ABSTRACT:** To sequestrate or scavenge ionic species in desiccated cells is one of the putative functions of group 3 late embryogenesis abundant (G3LEA) proteins. We still lack direct physicochemical information on how G3LEA proteins and their characteristic primary amino acid sequences, i.e., 11-mer motif repeats, behave in the presence of salts under water-deficit conditions. In the current study, we investigated salt effects as a function of water content on the structural and thermodynamic properties of the 22-mer peptide (PvLEA-22), consisting of two tandem repeats of the consensus 11-mer motif of G3LEA proteins from the larvae of *P. vanderplanki*. The results of circular dichroism (CD) and Fourier transform infrared (FT-IR) spectroscopic measurements indicate four main points as follows: (1) PvLEA-22 is in random coils in the aqueous solutions with or without a salt. (2) Dried PvLEA-22, whether salt-free or mixed with NaCl or KCl, is largely folded as  $\alpha$ -helix. (3) When dried with MgCl<sub>2</sub> or CaCl<sub>2</sub>, PvLEA-22 adopts  $\beta$ -sheet structure as well as random coil. (4) PvLEA-22 faithfully reproduces the conformational changes of the native LEA protein in response to added salts. Furthermore, through temperature-modulated differential scanning calorimetry (TMDSC) measurements, dried PvLEA-22 is found to be in the glassy state at ambient temperatures, independent of which salt is present. On the basis of these results, we discuss the intrinsic nature and putative functional roles of G3LEA proteins under salt-rich conditions.



Late embryogenesis abundant (LEA) proteins were initially discovered about 30 years ago in plant seeds where they are highly expressed at a late stage of maturation.<sup>1,2</sup> Several families of LEA proteins from many plant species are now widely recognized as key biological molecules for the acquisition of tolerance against various environmental stresses such as drought, freezing, and high-salt conditions.<sup>3–14</sup> In recent years LEA proteins have also been found in diverse animals<sup>15</sup> including nematodes,<sup>16–20</sup> bdelloid rotifers,<sup>21,22</sup> an African sleeping chironomid, *Polypedium vanderplanki*,<sup>23</sup> crustaceans,<sup>24</sup> and Collembola (springtails).<sup>25</sup> These LEA proteins belong to the so-called group 3 LEA (G3LEA) proteins: LEA proteins are known to be classified into several groups according to gene expression pattern or amino acid sequence.<sup>3–10,12–14</sup>

A defining feature of the primary structure of G3LEA proteins is the occurrence of repeated units of a loosely conserved 11-mer amino acid sequence.<sup>26</sup> An early study by Dure defined the consensus motif of the repeated 11-mer units of G3LEA proteins from plants as TAQAAGEKAGE.<sup>27</sup> Later he elaborated this consensus motif as “ΦΦΩXΦΨΩΨΦXΩ”, where Φ, Ω, and Ψ represent hydrophobic residues, negatively charged or amide residues, and positively charged residues, respectively, and X represents a nonspecifically conserved amino acid residue.<sup>28</sup> Recently, our research group used bioinformatics to elucidate the differences and similarities between the 11-mer consensus motif of G3LEA proteins of three different types of anhydrobiotic organism: Magnoliophyta

(i.e., flowering plants), nematodes, and *P. vanderplanki*.<sup>29</sup> The consensus motif of the repeated 11-mer units of G3LEA proteins in *P. vanderplanki*, for example, was determined to be AKDXTKEKAXE.

To date, several molecular mechanisms have been proposed to describe functional aspects of LEA proteins. One is that G3LEA proteins form intracellular filaments that act as a form of cytoskeleton in dehydrated cells through a desiccation-induced conformational shift from aqueous random coils to largely  $\alpha$ -helical structures.<sup>9</sup> *In vitro* experiments with Fourier-transform infrared (FT-IR) spectroscopic analysis indicated that a G3LEA protein from nematode (*Aphelenchus avenae*), AavLEA1,<sup>30</sup> and a plant mitochondrial G3LEA protein<sup>31</sup> both undergo structuralization to  $\alpha$ -helical coiled coils on dehydration from random coil in the aqueous state. FT-IR spectra of a dried G3LEA protein from pollen, called D-7 LEA, also exhibited  $\alpha$ -helical components which were absent in aqueous solution, although no vibrational peaks characteristic of coiled coils were observed in either condition.<sup>32</sup> Lately, we investigated the structural and thermodynamic properties of model peptides comprising two or four repeats of the consensus 11-mer motif characteristic of G3LEA proteins from Magnoliophyta, nematodes, and *P. vanderplanki*.<sup>29</sup> All

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model peptides were subjected to FT-IR measurements after complete deuterium exchange, and the results showed reversible structural transformation between random coil in solution and  $\alpha$ -helical coiled coils induced by desiccation.<sup>29</sup> Furthermore, conventional differential scanning calorimetry (DSC) indicated that the dried model peptides were in the glassy state up to at least 100 °C. In addition, from temperature-dependent FT-IR analysis, we observed that the 11-mer repeat units reinforced the glassy matrix of the disaccharide trehalose,<sup>29</sup> one of the compatible solutes that accumulate in anhydrobiotic organisms exposed to drought stress.<sup>33–35</sup> Furthermore, molecular dynamics simulations for a 66-amino acid fragment of the G3LEA protein,<sup>36</sup> which has at least four copies of the characteristic 11-mer G3LEA motif from nematode (*A. avenae*), indicated that hydrogen-bonding interactions were the primary factor determining fragment conformation and stability during dehydration. Thus, these experimental and theoretical studies using short peptides provide invaluable information about the intrinsic conformational nature of the 11-mer repeat units of G3LEA proteins.

Another putative function of G3LEA proteins is to sequester or scavenge ionic species in water-deficient cells, so that any undesirable effect of concentrated electrolytes, and concomitant elevated osmotic pressure, protein denaturation, etc., might be mitigated. This hypothesis was proposed by Dure III,<sup>27</sup> who used computer modeling to argue that the 11-mer motif repeating units of G3LEA proteins promote the formation of dimeric amphiphilic  $\alpha$ -helices in the presence of certain ionic species in dehydrated cells. Subsequently, various studies have demonstrated improved salt tolerance in microorganisms or plants overexpressing G3LEA proteins,<sup>37–44</sup> as summarized in Table S1 of the Supporting Information. For example, Zhang et al. found that the expression of barley G3LEA protein, HVA1, in yeast improved its resistance to salt stress.<sup>40</sup> Similarly, Liu et al. expressed the G3LEA protein, PM2, from soybean (*Glycine max*) in *Escherichia coli*, and showed that transformed bacteria were more tolerant of high-salt conditions than a control strain.<sup>37,38</sup> Furthermore, they constructed recombinant *E. coli* expressing the truncated polypeptide, PM2A (amino acid sequence 1–126 in PM2), PM2B (aa 129–262, containing repeats of a 22-mer sequence, itself comprising two variant 11-mer motifs), or an artificial polypeptide PM2C (two tandem repeats of the 22-mer repeat region).<sup>37</sup> Under salt stress, recombinant *E. coli* expressing either PM2A or PM2B had survival rates almost equal to that of bacteria containing the full-length LEA protein, PM2. In contrast, when subjected to salt stress, the survival rate of *E. coli* expressing PM2C was much higher than those of any other recombinant *E. coli* (i.e., with PM2, PM2A, or PM2B). These findings suggest that the 22-mer repeat region of the G3LEA protein, PM2, is the most effective at protecting host cells against salt stress.<sup>37</sup> However, it should be noted that in refs 37–44 none of the organisms used were exposed to severe water deficit typical of drying environments, and it is therefore not clear how far these results support the model of Dure III,<sup>27</sup> which depicts ion sequestration in the desiccated state.

While the available experimental evidence suggests an adaptive function of G3LEA proteins in salt stress tolerance, we still lack direct physicochemical information on how G3LEA proteins behave under high-salt conditions, especially at the low water activities typical of desiccation. In particular, studies focusing on the behavior of 11-mer motifs in the presence of

salts would be invaluable for testing the ion-scavenging hypothesis. Accordingly, we investigate here the properties of the 22-mer peptide (hereafter denoted as PvLEA-22 according to our previous paper<sup>29</sup>), consisting of two tandem repeats of the consensus 11-mer motif of G3LEA proteins from the larvae of *P. vanderplanki*.

## MATERIALS AND METHODS

**Peptides.** Two 22-mer peptides were used in this study. The first, PvLEA-22, comprised two repeats of the 11-mer consensus motif, AKDGTKEKAGE, characteristic of LEA proteins from *P. vanderplanki*. The second was a control peptide, with the amino acid sequence AKEKEGTDKAG-GAKDTGEKEKA, whose amino acid composition was identical to that of PvLEA-22, but its sequence was scrambled. Both PvLEA-22 and the control peptide were synthesized according to the solid phase method based on Fmoc chemistry,<sup>45</sup> followed by thorough purification by reverse phase HPLC (Hitachi L7000, Tokyo, Japan). The final products were identified by single peak appearance in the appropriate molecular weight position using MALDI-TOF mass spectrometry (Shimadzu KOMPACT MALDI III, Kyoto, Japan). The original peptides prepared in this way were lyophilized in Milli-Q water without any additives for frozen storage until use. Other details of these peptide preparations were described in our previous paper.<sup>29</sup>

**LEA Protein.** G3LEA protein from the larvae of *P. vanderplanki*, denoted as PvLEA2 in a previous study,<sup>23</sup> was purchased from Katakura Industry Co. in the freeze-dried form mixed with buffer salts (150 mM of NaCl and 20 mM of NaH<sub>2</sub>PO<sub>4</sub>). For desalting prior to use, the LEA protein was purified by repeated ultrafiltration until the conductivity of the filtered solution was as low as that of Milli-Q water. Subsequently, the purity of the LEA protein was checked by sodium dodecyl sulfate polyacrylamide gel electrophoresis. The purified LEA protein was lyophilized in Milli-Q water without any additives for frozen storage until use. For simplicity, hereafter the full length G3LEA protein from the larvae of *P. vanderplanki* will be referred to as the PvLEA protein, unless otherwise noted.

**Salts.** The following salts, purchased in the anhydrous form from Sigma Chemical Co., were used with model peptides or the LEA protein: NaCl, KCl, MgCl<sub>2</sub>, or CaCl<sub>2</sub>.

**Sample Preparation for Circular Dichroism (CD) Spectroscopy.** CD was performed on solutions in Milli-Q water of the 22-mer peptides (1 mg mL<sup>−1</sup>) and the PvLEA protein (0.5 mg mL<sup>−1</sup>). For binary mixtures with a selected salt, the molar ratios of salt per amino acid residue of the peptide or protein were 1 or 10, respectively.

**Dry Sample Preparation for FT-IR Spectroscopy.** Dried samples of (A) PvLEA-22 and control peptides, (B) PvLEA protein, and (C) droplets of selected aqueous salt solutions were prepared for FT-IR spectroscopy as follows:

- Each 22-mer peptide was dissolved in Milli-Q water at 2 wt %. We also prepared binary mixtures of each peptide with a selected salt where the molar ratios of the added salt per amino acid residue of the peptide were 0, 0.5, and 1.0. When the added salt is NaCl and its corresponding molar ratio is 0.5, for instance, this sample will be hereafter denoted as NaCl [0.5]. The dried peptide sample was obtained by placing an aliquot (10  $\mu$ L) of the

above peptide solution on a Teflon plate in a glass desiccator with relative humidity (RH) of 5% with silica gels at 25 °C.

- (B) Since the amount of PvLEA protein prepared was much smaller than that of the chemically synthesized model peptides, we obtained its dried sample in a slightly different way. The PvLEA protein was dissolved in Milli-Q water at 0.1 wt %, and then a selected salt was added so that the molar ratio of the salt per amino acid residue of the PvLEA protein was 1:1. Volumes of up to 30  $\mu$ L of the aqueous PvLEA protein solution were placed on a circular-shaped  $\text{CaF}_2$  infrared window ( $2 \times 20$  mm) in a glass desiccator (RH 5%) and left to dry.
- (C) To control for any effect of the added salts on the above dried samples, we determined the FT-IR spectrum of a dried sample of each pure salt: aliquots of a 0.2 M salt solution were dried on a Teflon plate or a  $\text{CaF}_2$  plate in a desiccator at 5% RH.

**Dry Sample Preparation for Temperature-Modulated Differential Scanning Calorimetry (TMDSC).** Thermodynamic experiments using TMDSC were carried out on dried PvLEA-22 samples prepared in the same way as for FT-IR measurements (A, above).

**Modification of Moisture Content of Samples for FT-IR Spectroscopy and TMDSC.** To examine the water content dependence of the molecular structure and thermodynamic properties of the 11-mer motif repeat units, several pieces of a dried PvLEA-22 sample in the form of thin film were placed on a  $\text{CaF}_2$  plate (20 mm (diameter)  $\times$  2 mm (thickness)) and then allowed to absorb moisture by exposure to various RH environments at room temperature (ca. 25 °C) for a selected period in a glass desiccator. Humidity was controlled with saturated aqueous solutions of  $\text{MgCl}_2$ ,  $\text{Mg}(\text{NO}_3)_2$ , NaCl, KCl, or  $\text{KNO}_3$ , which provided 38% RH, 60% RH, 75% RH, 85% RH, or 93% RH, respectively. The PvLEA-22 sample was weighed before and after treatment to estimate the amount of moisture uptake. After treatment, the resulting samples on  $\text{CaF}_2$  plates were used as such for FT-IR measurements, whereas for TMDSC the samples were transferred to aluminum DSC pans (TA Instruments, New Castle, DE).

**Water Contents of Dried Samples.** Water contents were determined by thermogravimetry: samples were weighed before and after heating at 170 °C for at least 20 min.

**CD Measurements.** CD spectra were recorded with a CD spectropolarimeter (JASCO, Tokyo, Japan) using a 0.2 mm path-length cell over the 180–250 nm range at room temperature.

**FTIR Measurements.** FTIR spectra were measured using a Fourier transform infrared spectrometer (JASCO FTIR 6100 and IMV-4000, Tokyo, Japan) equipped with a high-sensitivity mercury–cadmium telluride detector. Samples dried at 5% RH were pressed at  $\sim 240$  kg/cm<sup>2</sup> between two KBr plates ( $7 \times 7 \times 1$  mm). Model peptides of interest placed on a  $\text{CaF}_2$  plate subjected to the above moisture treatments were sandwiched with another  $\text{CaF}_2$  plate, with a Teflon spacer (50  $\mu$ m thickness) if necessary. All spectra were obtained in the range 700–4000 cm<sup>−1</sup> with a spectral resolution of 4 cm<sup>−1</sup> and 128 scans. In order to investigate the secondary structural properties of the model peptides and the PvLEA protein, the amide I and amide II absorption bands between 1500 and 1720 cm<sup>−1</sup> were

analyzed. Spectral manipulations for second-derivative spectra, Fourier-self-deconvolution, and curve fittings were performed using the JASCO (Tokyo, Japan) Spectra Manager Version 2 software.

**TMDSC Measurements.** All measurements were carried out using a Q-100 instrument equipped with modulation capability and a cooling accessory (TA Instruments, New Castle, DE). TMDSC has several advantages over normal DSC, the most important point being that so-called reversing and nonreversing thermal events can be detected on separate heat-flow curves.<sup>46–49</sup> Glass transitions, being thermally reversible phenomena, are differentiated from irreversible changes, such as recrystallization, devitrification, and heat-induced change in morphology of the measured sample. These technical advantages aid the interpretation of the origin of thermal phenomena observed. The apparatus was calibrated using indium and sapphire. For all TMDSC measurements, an underlying scan rate of 5 K min<sup>−1</sup> was used with a temperature amplitude of 0.8 °C over a period of 60 s. These parameter values allowed at least four modulation cycles per glass transition temperature, sufficient for differentiation of reversing and nonreversing events.<sup>50</sup> An aluminum DSC pan was used for both dried and moisture-treatment samples.

Thermal events recorded were analyzed using Universal Analysis software (TA Instruments). Glass transition temperatures are described below as their onset values, which were defined to be the intersection of the extrapolated baseline with the tangent at the inflection point in the stepwise change in heat capacity.

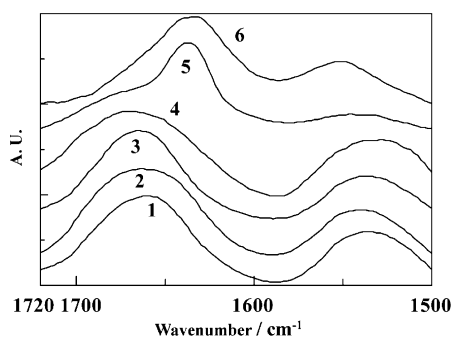
## RESULTS

**Structure of LEA Model Peptides in Solution and the Dry State.** PvLEA-22 in aqueous solution exhibited a CD spectrum with a single minimum around 200 nm, characteristic of random coil structure (data not shown), and this spectral feature was unchanged by addition of a salt (NaCl, KCl,  $\text{MgCl}_2$ , or  $\text{CaCl}_2$ ) up to 10-fold molar ratio per amino acid residue of the peptide (data not shown). Similar results were obtained for the control peptide. These results suggest that PvLEA-22 and the control peptide both adopt predominantly random-coil structures in aqueous solution irrespective of the presence or absence of salt.

For the dried solids of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ , their FT-IR spectra showed clear absorbance in the neighborhood of 1625–1630 cm<sup>−1</sup> (data not shown), just within the amide I region. It has been reported that the appearance of such absorbance is characteristic of hydrated crystals of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ .<sup>51</sup> In contrast, no such FT-IR peaks were observed for desiccated NaCl and KCl. Fortunately, none of these dried salts exhibited peaks around the amide II region (1580–1520 cm<sup>−1</sup>).

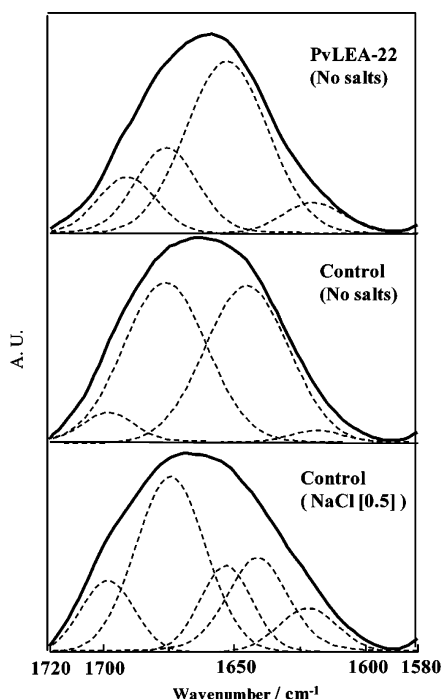
Figure 1 shows the FT-IR spectra in a region from 1500 to 1720 cm<sup>−1</sup> for several dried 22-mer peptide samples. All spectra have two peaks, which are in the amide I and II regions, respectively, although their relative intensity (amide I/amide II) varies from spectrum to spectrum. In particular, the relative intensities for the spectra 5 and 6, which were measured for the samples including  $\text{MgCl}_2$  and  $\text{CaCl}_2$ , respectively, are apparently larger than those of the other spectra. This is most likely due to the overlap of the absorbance of the hydrate crystal with that of the amide I band of the peptide.





**Figure 1.** Amide I and II regions of FT-IR spectra for the following samples in the dried state. 1: PvLEA-22 without salt; 2: the control peptide without salt; 3: PvLEA-22 mixed with NaCl [0.5]; 4: the control peptide with NaCl [0.5]; 5: PvLEA-22 with MgCl<sub>2</sub> [0.5]; 6: PvLEA-22 with CaCl<sub>2</sub> [0.5]. 0.5 in the brackets indicates the molar ratio of the added salt per amino acid residue of the peptide, as appropriate.

Figure 2 (top) shows the amide I spectrum and its constitutive spectral components for salt-free PvLEA-22 in



**Figure 2.** Amide I region of the FT-IR spectrum. The original absorbance curve measured is shown as a solid line. Its constitutive spectral components are shown as broken lines, as evaluated by Fourier self-deconvolution followed by curve fitting. The number and peak positions of these components were determined from the second-derivative spectra (not shown) of the original absorbance curve.

the dry state. Clearly, the peak at 1653 cm<sup>-1</sup>, assignable to  $\alpha$ -helix,<sup>52–56</sup> is the major component with a relative area up to about 60% (see the data for sample A in Table 1). When aqueous PvLEA-22 mixed with either NaCl or KCl was dried (samples from B to E in Table 1), its overall spectral profile in the amide I region was little changed compared with that shown in Figure 2 (top). However, the maximal spectral component of the amide I band, corresponding to  $\alpha$ -helix,

shifted slightly to a higher wavenumber on addition of NaCl or KCl. This suggests that details in the  $\alpha$ -helical structure of dried PvLEA-22 were somewhat modified by the salt. Aside from this subtle difference, the LEA model peptide in the dried state has  $\alpha$ -helix rich structure, irrespective of the absence or presence of either NaCl or KCl.

We also obtained the amide I spectrum and its constitutive spectral components for the dried control peptide without salts (Figure 2 (middle)). In this case, there are two spectral components at 1643 and 1676 cm<sup>-1</sup>, both having a relative area as large as 46% (a sample F in Table 1). The former and latter peaks are located in the wavenumber regions characteristic of random coil and  $\beta$ -turn structures, respectively.<sup>52–56</sup> On the other hand, when the control peptide was desiccated in the presence of NaCl [0.5] (sample G in Table 1), its amide I spectrum (Figure 2 (bottom)) exhibited an additional spectral component at 1653 cm<sup>-1</sup>, corresponding to  $\alpha$ -helix structure.<sup>52–56</sup> Furthermore, the area of this additional peak increased, although slightly, from 16% to 24% when the concentration of NaCl was increased (see sample H in Table 1). When the added salt was KCl [0.5] or KCl [1.0], similar trends were observed (data for samples I and J in Table 1). These spectroscopic information indicates that the added salts studied may induce a partial structural shift toward  $\alpha$ -helix in the control peptide.

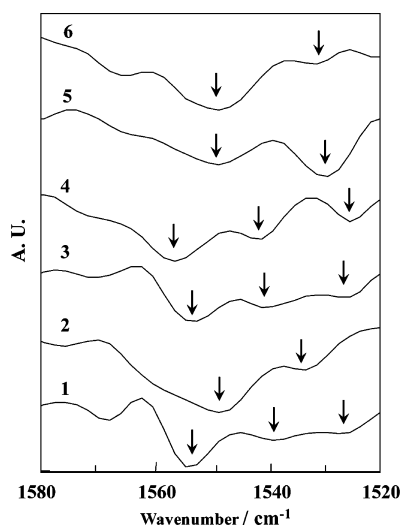
It should be noted that there are two additional spectral components for the amide I band of samples A–J: one is below 1625 cm<sup>-1</sup> and the other above 1690 cm<sup>-1</sup>. These peaks have been reported for denaturated and/or aggregated proteins resulting from drying,<sup>57</sup> heating,<sup>57–59</sup> or mixing with alcohol.<sup>60</sup> The appearance of these peaks, albeit as minor components, implies that PvLEA-22 underwent a degree of desiccation-induced denaturation and/or aggregation.

As described above, for PvLEA-22 desiccated with MgCl<sub>2</sub> or CaCl<sub>2</sub>, there is a possibility that the FT-IR absorbance of the hydrate crystal of the added salt overlaps with the amide I band of the peptide, preventing us from correctly interpreting the spectrum. Therefore, in these dried peptide samples we used the amide II band for secondary structural analysis. However, the width of the amide II band is narrower than that of the amide I band, making the spectral region characteristic of each secondary structure less well-separated than the amide I band. For the amide II region, there is relatively little information in the literature on the relationship between its spectral components and the corresponding secondary structures of samples tested.<sup>52–56</sup> In the present study, the spectral components in the amide II band were assigned by use of the peptides whose conformations are already known from the amide I band analysis. Figure 3 shows the second-derivative spectra of the amide II region in the spectra from 1 to 6 given in Figure 1. Spectra 1 and 3, from the dried PvLEA-22 samples, each of which had large  $\alpha$ -helical content (Table 1), show a peak at ca. 1540 cm<sup>-1</sup>. In contrast, no such peak was detected for the dried salt-free control peptide (spectrum 2), which had no  $\alpha$ -helical structure (Table 1). The control peptide desiccated with NaCl gave a peak around 1540 cm<sup>-1</sup> (spectrum 4), adopting a partial  $\alpha$ -helical structure (Table 1). On the basis of these findings, the spectral component at ca. 1540 cm<sup>-1</sup> in the amide II band is likely to represent  $\alpha$ -helix. Other peaks in the vicinity of 1525 and 1555 cm<sup>-1</sup> are in turn assignable to  $\beta$ -sheet and random coil, respectively, with reference to available data from the literature.<sup>52–56</sup>

**Table 1. Peak Positions ( $\text{cm}^{-1}$ ) of the Spectral Components of the Amide I Band and Their Relative Areas (%)<sup>a</sup> in Model Peptides Dried with or without a Selected Salt**

sample name	chemical components <sup>b</sup>	spectral components									
		cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%		
	PvLEA-22										
A	no salts	1620	8	N.D. <sup>c</sup>	1653	57	1676	22	1691	14	
B	NaCl [0.5]	1620	4	N.D.	1654	63	1674	22	1693	12	
C	NaCl [1.0]	1620	3	N.D.	1658	70	1675	13	1693	12	
D	KCl [0.5]	1620	4	N.D.	1654	60	1675	23	1695	13	
E	KCl [1.0]	1620	4	N.D.	1658	69	1676	18	1695	8	
	control										
F	no salts	1618	2	1643	46	N.D.	1676	47	1697	5	
G	NaCl [0.5]	1622	9	1641	22	1653	16	1674	42	1698	13
H	NaCl [1.0]	1622	11	1644	26	1658	24	1676	21	1693	18
I	KCl [0.5]	1626	9	1641	14	1653	22	1678	41	1697	15
J	KCl [1.0]	1625	9	1644	11	1658	28	1676	35	1697	17

<sup>a</sup>Numerical data of relative areas were rounded to one decimal place, and thereby the sum of the relative areas over all amide I components is not necessarily 100%. <sup>b</sup>0.5 and 1.0 in the brackets indicate the molar ratios of the added salt per amino acid residue of the each peptide. <sup>c</sup>Not detected.



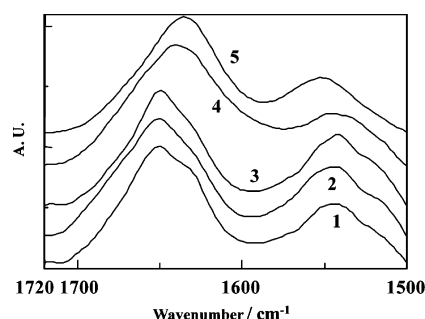
**Figure 3.** Amide II region of the second-derivative spectra. The labeled number for each spectrum and its corresponding sample are the same as in Figure 1. The arrows show the peak positions of interest in the given spectra.

For PvLEA-22 dried with either  $\text{MgCl}_2$  [0.5] or  $\text{CaCl}_2$  [0.5], the corresponding second-derivative spectrum for the amide II band (spectrum 5 or 6 in Figure 3) indicates that there is no peak near  $1540 \text{ cm}^{-1}$ , while clear peaks were detected in the neighborhood of  $1530$  and  $1550 \text{ cm}^{-1}$ . With increasing concentrations of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ , these spectroscopic features remained almost unchanged (data not shown). Furthermore, similar spectral behavior was also observed for the dried binary mixtures of the control peptide with either  $\text{MgCl}_2$  or  $\text{CaCl}_2$ , regardless of salt concentration. These results suggest that both PvLEA-22 and the control peptide are unlikely to adopt  $\alpha$ -helical structure in the dry state in the presence of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ . Taken together with the results for the mixtures with NaCl or KCl, the conformational properties of PvLEA-22 and the control peptide in the dried state seem strongly dependent on the ionic valency of the salt added.

**Structure of PvLEA Protein in Solution and the Dry State.** CD spectra of an aqueous solution of pure PvLEA protein showed a strong negative band at  $200 \text{ nm}$  as well as a

relatively weak one around  $220 \text{ nm}$  (data not shown), being typical of other G3LEA proteins<sup>22,30,31</sup> and more generally for largely unfolded proteins.<sup>55,56</sup> Similar CD spectral features were observed for all binary aqueous solutions of the PvLEA protein mixed with the salts NaCl, KCl,  $\text{MgCl}_2$ , or  $\text{CaCl}_2$ . These results indicate that the PvLEA protein adopts a random coil configuration in aqueous solution irrespective of the amount and type of added salt in the concentration range tested (data not shown).

The FT-IR spectrum of dried PvLEA protein exhibited strong dependence on added salt, however, as shown in Figure



**Figure 4.** Amide I and II regions of FT-IR spectra for the following samples of the PvLEA protein in the dried state. 1: no salt; 2: with NaCl [1.0]; 3: with KCl [1.0]; 4: with  $\text{MgCl}_2$  [1.0]; 5: with  $\text{CaCl}_2$  [1.0]. 1.0 in the brackets indicates the molar ratio of the added salt per amino acid residue of the PvLEA protein.

4. The results of detailed analysis of the amide I band of spectra from 1 to 3 are given in Table 2 (sample name a, b, and c), together with the corresponding data of PvLEA-22 in the dried state for comparison. Every sample (a, b, and c) has the major component at  $1652$  or  $1653 \text{ cm}^{-1}$ , typical of  $\alpha$ -helical structure.<sup>52–56</sup> Together with the results of the CD spectra for the solution state, it can safely be said that the PvLEA protein itself undergoes a conformational change upon desiccation from random coil to  $\alpha$ -helix, and such a change also occurs in the presence of NaCl [1.0] or KCl [1.0].

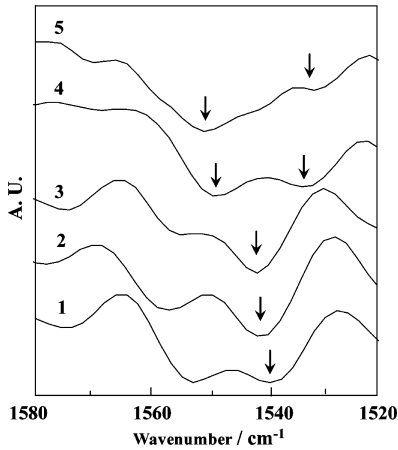
Spectra 4 and 5 in Figure 4 exhibit larger amide I/amide II intensity ratios than those of spectra 1 to 3, which is due to overlapping of the absorbance from the PvLEA protein and that

**Table 2. Peak Positions ( $\text{cm}^{-1}$ ) of the Spectral Components of the Amide I Band and Their Relative Areas (%)<sup>a</sup> in the PvLEA Protein and PvLEA-22 Dried with or without a Selected Salt**

sample	chemical components	spectral components							
		cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%
	PvLEA protein								
a	no salts	1629	34	1653	48	1678	16	1697	3
b	NaCl [1.0]	1630	23	1652	54	1678	22	1697	1
c	KCl [1.0]	1630	32	1653	53	1678	14	1698	2
	PvLEA-22 <sup>b</sup>								
A	no salts	1620	8	1653	57	1676	22	1691	14
C	NaCl [1.0]	1620	3	1658	70	1675	13	1693	12
E	KCl [1.0]	1620	4	1658	69	1676	18	1695	8

<sup>a</sup>See the footnote *a* of Table 1. <sup>b</sup>Cited from Table 1.

from the hydrate crystals of the added salt,  $\text{MgCl}_2$  or  $\text{CaCl}_2$ . Therefore, as for PvLEA-22, the secondary structure of the dried PvLEA protein in the presence of  $\text{MgCl}_2$  [1.0] or  $\text{CaCl}_2$  [1.0] was inferred from amide II band analysis. This suggests that the PvLEA protein dried with  $\text{MgCl}_2$  [1.0] or  $\text{CaCl}_2$  [1.0] contains no appreciable  $\alpha$ -helical component because spectra 4 and 5 in Figure 5 have no peak in the neighborhood of 1540

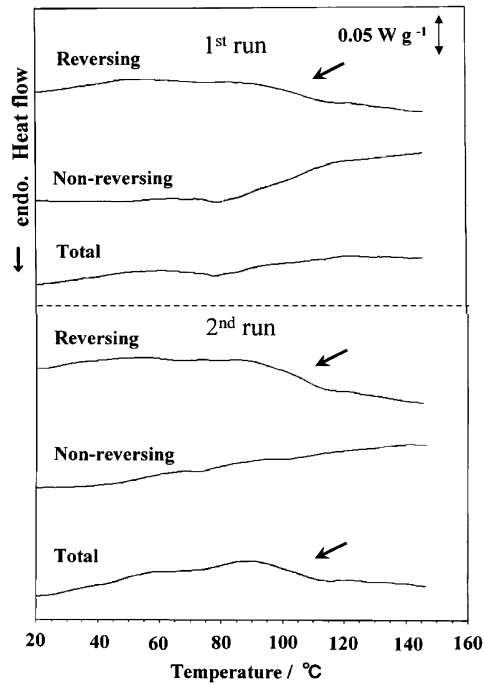


**Figure 5.** Amide II region of the second-derivative spectra. The labeled number for each spectrum and its corresponding sample are the same as in Figure 4. The arrows show the peak positions of interest in the given spectra.

$\text{cm}^{-1}$ . The peaks around 1530 and 1550  $\text{cm}^{-1}$  observed in these spectra inform us of the presence of  $\beta$ -sheet and random coil structures, respectively. These spectral features are in agreement with those of dried PvLEA-22 in the presence of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ .

Taken together, the PvLEA protein undergoes dehydration-induced structural changes in a similar way to PvLEA-22. In other words, the 22-mer model peptide, PvLEA-22, faithfully reproduces the structural behavior of the full-length LEA protein.

**Glass Transition of PvLEA-22 in the Dried State.** Figure 6 (upper half) shows the TMDSC thermogram in the first heating run of PvLEA-22 dried with KCl [1.0]. In general, reversing phenomena such as glass transitions are reflected in the reversing component of the TMDSC thermogram obtained. As can be seen from Figure 6 (upper half), the reversing component curve clearly exhibited a baseline shift starting around 90 °C, suggesting the occurrence of a glass transition. In



**Figure 6.** TMDSC thermograms in the first and second heating runs for PvLEA-22 dried with KCl [1.0]. The arrow indicates the glass transition.

addition, as shown in Figure 6 (lower half), the second heating run provided the reversing component curve almost identical to that in the first heating run. This observation strongly supports the above interpretation. However, it may be necessary here to explain why the total heat flow curve, which corresponds to the heat flow curve obtained by conventional DSC,<sup>46–49</sup> exhibited no glass-transition-like behavior in the first run but did show such behavior in the second heating scan. In the first run, the total heat flow curve is very similar to the nonreversing component curve, which implies that the thermal event of the first heating run is dominated by any nonreversing thermal events. For the second run, such unfavorable thermal events apparently do not occur, which is suggested by the fact that the nonreversing component curve was essentially a simple straight line with a slight ascendant slope. As a result, the total heat flow curve mainly reflected the reversing events in the second heating run and exhibited a clear glass transition similar to that in the reversing component curve.

Table 3 summarizes the glass transition temperatures for the dried PvLEA-22 mixed with a selected salt. All samples are in

**Table 3. Glass Transition Temperatures ( $T_g$ )<sup>a</sup> of Dried PvLEA-22**

sample	chemical component	$T_g$ (°C)	water content <sup>b</sup>
A	no salts	108	2
B	NaCl [0.5]	99	2
C	NaCl [1.0]	95	4
D	KCl [0.5]	98	2
E	KCl [1.0]	92	4
K	MgCl <sub>2</sub> [0.5]	58	9
L	MgCl <sub>2</sub> [1.0]	55	15
M	CaCl <sub>2</sub> [0.5]	63	5
N	CaCl <sub>2</sub> [1.0]	51	8

<sup>a</sup>Obtained as onset values in °C from the reversing component curve of TMDSC. <sup>b</sup>The water contents shown were defined on a dry total mass basis and given in wt %.

the glassy state at room temperature, although their  $T_g$  values are strongly dependent on the choice of added salt. All  $T_g$  values in Table 3 were somewhat lower than that of PvLEA-22 dried without salt at 108 °C. Samples from K to N provided  $T_g$  values lower by ca. 30 or 40 °C than those from B to E. There are two possible factors for such lowering of  $T_g$ . One is the water content in the sample of interest. Residual water acts as a plasticizer and thereby depresses the glass transition temperature of the sample.<sup>61</sup> Samples from K to N contain the hydrate crystals of the added salt concomitantly precipitated on desiccation, and thereby the residual water from the hydrate crystal may modify  $T_g$ . The other possible physicochemical factor is the secondary structure of the peptide. Unlike samples from B to E, no  $\alpha$ -helical secondary structure was detected in PvLEA-22 for samples from K to N, where only  $\beta$ -sheet and random coil structures were detected as described above. Such a structural difference of PvLEA-22 seems to bring about a significant different in its molecular motion in the dry state, probably leading to the different  $T_g$  values.

**Water Content Dependence of Molecular Structure and Glass Transition of PvLEA-22.** Table S2 in the Supporting Information summarizes the data for the water content dependence of several physicochemical parameters of salt-free PvLEA-22. It should be mentioned that the lower limit of the temperature range measurable in the present TMDSC experiments was −50 °C, which was determined by the cooling accessory available. The points to be noted in Table S2 are as follows:

- (1)  $\alpha$ -Helical secondary structure was detected at room temperature until the water content increased to at least 20 wt %, but it disappears when the water content reached as high as 30 wt %, where roughly 2.4 water molecules are present around one amino acid residue of PvLEA-22.
- (2)  $T_g$  values were sensitive to the water content: for example, it reduces to −10 °C from 108 °C with an increase in water content from 2 to 7 wt %. When the water content increased to 20 wt %, two glass transitions appeared, suggestive of the occurrence of phase separation during cooling.
- (3) According to the  $T_g$  values observed, the PvLEA-22 samples with water contents of 7, 9, 12, 15, and 20 wt %

are in the rubbery state at room temperature. In addition, FT-IR spectra measured at room temperature indicated that the rubbery peptide chains partially adopt  $\alpha$ -helical structure.

### Water Content Dependence of Molecular Structure and Glass Transition of PvLEA-22 Mixed with NaCl.

Tables S3 and S4 in Supporting Information summarize the data for the water content dependence of several physicochemical parameters of PvLEA-22 in the presence of NaCl [0.5] and NaCl [1.0], respectively. The points to be noted are as follows:

- (1)  $\alpha$ -Helical secondary structure was observed for samples with NaCl [0.5] and NaCl [1.0] until their water contents increased to 20 and 30 wt %, respectively. It was no longer observed when the water content reached 40 wt %.
- (2)  $T_g$  values decreased steeply with increasing water content. As in the case of the salt-free sample, two glass transitions were observed for the sample with NaCl [0.5] when the water content was increased to 23 wt %.

## DISCUSSION

Despite increasing evidence to suggest a protective function of G3LEA proteins against salt stress, direct physicochemical information on how G3LEA proteins behave under high-salt conditions, especially at low water activities associated with desiccation, has been lacking. In particular, the structural and thermodynamic properties of the 11-mer motif units in the presence of salts have not been examined so far although they may provide essential information for testing the ion-scavenging hypothesis. In the current study, we investigated the effects of each salt selected from four candidates (NaCl, KCl, MgCl<sub>2</sub>, or CaCl<sub>2</sub>) not only on the structural features of PvLEA-22, the control peptide, and the parent LEA protein but also on the thermodynamic properties of PvLEA-22. The results provided answers to the following questions: (1) What structures do the 11-mer repeats unique to G3LEA proteins adopt in the presence of salts in the dry state? (2) To what extent can the short model peptide, PvLEA-22, reproduce the structural properties of the parent G3LEA protein? (3) Do the 11-mer repeat units vitrify in the presence of salts in the dry state? In other words, are two of the mechanisms hypothesized to contribute to desiccation tolerance, namely vitrification (of the cytoplasm) and ion scavenging by LEA proteins, compatible with each other? (4) How do the 11-mer motifs also exert protective effects against salt stress even at relatively high water activities, i.e., not just at the later, extremely dry, stages of the desiccation process?

Clear-cut answers to question (1) were obtained from the results of CD and FT-IR spectroscopy for the PvLEA-22 and control peptides. In aqueous solution, both peptides were unstructured irrespective of the presence or absence of selected salts as indicated by their CD spectra. On the other hand, the FT-IR measurements indicated that dried PvLEA-22 adopts  $\alpha$ -helical structure whether or not it was mixed with NaCl or KCl (samples from A to E in Table 1). The control peptide was found to be unstructured in the salt-free dried state but underwent a degree of structural transformation to  $\alpha$ -helices on dehydration in the presence of NaCl or KCl. In these desiccation-induced structural changes, Na<sup>+</sup> and K<sup>+</sup> ions seem to be of primary importance rather than the anion Cl<sup>−</sup>, since the addition of MgCl<sub>2</sub> or CaCl<sub>2</sub> induced different



structural changes: no  $\alpha$ -helices were recognized, and instead  $\beta$ -sheet structure was detected. The above result indicates that the dried control peptide is also able to sequester ions. It should be pointed out, however, that the  $\alpha$ -helical content was at most 20 wt % (Table 1), which is less than that of the dried PvLEA-22: the control peptide may be less able to scavenge ions than PvLEA-22. In other words, the amino acid sequence, not just amino acid composition, is an important determinant of ability to act as an ion scavenger, at least for  $\text{Na}^+$  and  $\text{K}^+$ .

In the presence of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ , both PvLEA-22 and the control peptide were shown to adopt  $\beta$ -sheet structure together with random coil in the dry state. Two different mechanisms are conceivable to explain the  $\beta$ -sheet formation. One is the intramolecular coordination of the divalent cation ( $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$ ) by negatively charged side chains like the dissociated carboxyl groups ( $\text{COO}^-$ ) on the amino acid residues, Asp or Glu. The other is the intermolecular bridge formation through the electrostatic interaction between the divalent cation and  $\text{COO}^-$  groups, for instance. PvLEA-22 and the control peptide dried with  $\text{MgCl}_2$  or  $\text{CaCl}_2$  exhibited almost identical FT-IR spectroscopic features. Such minor dependence on the amino acid sequence implies that intermolecular, rather than intramolecular, factors are most important in the formation of  $\beta$ -sheet.

To address question (2), we performed spectroscopic measurements for the PvLEA protein as well as the model peptides. Consequently, it was found that the native LEA protein was in the random coil state in aqueous solution with or without a selected salt, consistent with previous reports for other G3LEA proteins.<sup>22,30,31</sup> The molecular structure of the dried PvLEA protein varied depending on the type of salt added, in a generally similar fashion to the peptide PvLEA-22, which therefore can accurately mimic the conformational response of the native LEA protein to the presence of salt. As a whole, our results have the following implications for the ion-scavenger hypothesis.<sup>27</sup> First, that the ability of 11-mer repeat units to sequester  $\text{Na}^+$  and  $\text{K}^+$  ions may depend on the amino acid sequence of the motif, rather than simply its amino acid composition, and second, that the ion-scavenger hypothesis overlooks the possibility of  $\beta$ -sheet formation of the 11-mer motifs, triggered by intermolecular bridge formation via the divalent cations targeted for sequestration.

In regard to question (3), TMDSC measurements were carried out for dried binary mixtures of PvLEA-22 and a selected salt. Every sample examined was in the glassy state at ambient temperature as demonstrated by  $T_g$  values, which were sensitive to the choice of the mixed salt (Table 3). These findings suggest that the two well-known hypotheses on anhydrobiotic mechanisms, i.e., vitrification and ion scavenging, are compatible with each other, at least as far as the repeating moieties of the 11-mer-motif unique to G3LEA proteins are concerned. Our results also have an important implication for the drought-stress tolerance of *P. vanderplanki*. In nature its dried-up larvae, with a water content as low as 3 wt %, can survive the dry season in dried mud on rocks, despite the rock surface temperature reaching 60 °C in the sun.<sup>61</sup> The  $T_g$  values of samples B to E (Table 3) are consistent with maintenance of the glassy state, even under the severe conditions *P. vanderplanki* experiences in the environment.

According to refs 37–44, the expression of a G3LEA protein results in increased salt tolerance in a variety of organisms. In every case reported, however, the organism was not subjected

to desiccation during the course of the experiments. In this context the enhanced tolerance against salt stress is not simply explained by the ion-sequestration mechanism for the dried state; hence question (4) above. To address this, we examined the structural and thermodynamic properties of PvLEA-22 with and without a selected salt as a function of water content. As shown in Table S2 of the Supporting Information,  $\alpha$ -helical secondary structure was no longer detected for salt-free PvLEA-22 when the water content increased to 30 wt %, but was present with water content below 20 wt %. The water contents of 30 and 20 wt % correspond to 2.4 and 1.4 water molecules per amino acid residue of PvLEA-22 on average, respectively. It is of interest here to compare the present finding with that from molecular dynamics (MD) simulations of a 66-mer peptide from a nematode G3LEA protein in ref 36. According to this simulation, the secondary structure is predominantly random coil at a water content of ca. 20 wt % (105 water molecules per 66-mer peptide), whereas the fractional representation of  $\alpha$ -helices exceeds that of random coils when the residual water falls to 10 wt % (46 water molecules per the 66-mer peptide) or below. Thus, with respect to the threshold of water content below which  $\alpha$ -helix predominates, there is good agreement between the experimental data for PvLEA-22 and the computational results for the 66-mer nematode peptide. This suggests that similar parameters are driving  $\alpha$ -helix formation for both PvLEA-22 and the 66-mer peptide. The MD simulation study suggested that the dehydration-induced conformational change of the 66-mer peptide is primarily due to intramolecular hydrogen-bonding interactions followed by electrostatic interactions.<sup>36</sup>

In the presence of salts, however, the correlation between water content and the conformational properties of PvLEA-22 is not as straightforward since at least three physicochemical factors need to be considered, i.e., the degree of dissociation of the added salt, hydration number of the dissociated ions, and the percentage of ions undergoing electrostatic interaction with PvLEA-22. As shown in Tables S3 and S4 of the Supporting Information, for the binary mixtures of PvLEA-22 with NaCl [0.5] and NaCl [1.0], no  $\alpha$ -helical secondary structure was detected when their water contents were  $\geq 40$  wt %. If the total NaCl in these samples were perfectly dissociated into  $\text{Na}^+$  and  $\text{Cl}^-$ , with hydration numbers of 4 and 6, respectively,<sup>62</sup> 110 and 220 mol of water would be required for the hydration of every ion (i.e., the sum of  $\text{Na}^+$  and  $\text{Cl}^-$  ions) in the samples of PvLEA-22 with NaCl [0.5] and NaCl [1.0], respectively. This is more than the total amount of water in these samples, i.e., 105 and 129 mol (Tables S3 and S4), respectively. Therefore, since there is insufficient water to completely dissolve the salt,  $\text{Na}^+$  and/or  $\text{Cl}^-$  ions may be forced to bind to charged side chains of the amino acid residues of PvLEA-22 or to crystallize out of solution. Such a crystallization was, however, not observed and thereby PvLEA-22 seems likely to sequester ions even in the disordered conformational state. This might then explain the correlation between the expression of a selected G3LEA protein or its related polypeptide and the enhanced tolerance against salt stress without desiccation.<sup>37–44</sup>

In our previous study,<sup>29</sup> deuterated PvLEA-22 (PvLEA-22 (D)) and control peptide (control peptide (D)) were used to perform FT-IR spectroscopy in both aqueous and dried states (without salts). It is noteworthy that PvLEA-22 (D) in the dried state formed  $\alpha$ -helical coiled coils, as indicated by the well-resolved triplet band in the amide I region typical for such

deuterated structures: ca. 1626–1628  $\text{cm}^{-1}$ , ca. 1638–1640  $\text{cm}^{-1}$ , and ca. 1652–1653  $\text{cm}^{-1}$ .<sup>63,64</sup> However, as described above, dried PvLEA-22 without H-D exchange exhibited predominantly a single band at the wavenumber position typical of  $\alpha$ -helix. Such deuteration effects on the amide I band of PvLEA-22 are in good agreement with those for coiled coil proteins and are likely due to the different strength of hydrogen bonds in  $\text{H}_2\text{O}$  and  $\text{D}_2\text{O}$ .<sup>63,64</sup> In Table S5 of the Supporting Information, we provide data of band positions and relative areas of the FT-IR spectra for PvLEA-22 (D) and the control peptide (D) dried with a selected salt. These observations can be summarized as follows:

- (1) Both PvLEA-22 (D) and the control peptide (D) were disordered in  $\text{D}_2\text{O}$  solutions, irrespective of the presence or absence of any salt. Furthermore, all of the desiccation-induced structuralization described below from (2) to (4) returned to random coils on rehydration.
- (2) PvLEA-22 (D) mixed with NaCl or KCl in the dried state adopted  $\alpha$ -helical coiled coils, as without salts.
- (3) Unlike in the absence of salts, the control peptide (D) was largely folded as  $\alpha$ -helical coiled coil when dried in the presence of NaCl or KCl.
- (4) On addition of  $\text{MgCl}_2$  or  $\text{CaCl}_2$ , both PvLEA-22 (D) and the control peptide (D) exhibited a tendency to form  $\beta$ -sheet structure in the dried state.

In summary, as a whole, the conformational responses of these deuterated model peptides to the addition of salts were consistent with those of the same peptides without H–D exchange.

## CONCLUSION

In the present study, we compared the effects of salts on the structural properties of the 11-mer repeat units typical of G3LEA proteins with effects on the native G3LEA protein itself and obtained information on the thermodynamic features of the 11-mer repeats. First, our FT-IR spectroscopic measurements demonstrated the following: (1) Dried PvLEA-22 adopted mainly  $\alpha$ -helical secondary structure in the presence or absence of NaCl or KCl. (2) The control peptide dried with NaCl or KCl underwent in part a structural shift to  $\alpha$ -helix, while the salt-free control peptide maintained a random coil conformation even on desiccation. (3) For both PvLEA-22 and the control peptide,  $\beta$ -sheet structure was induced by dehydration of their binary mixtures with  $\text{MgCl}_2$  or  $\text{CaCl}_2$ . (4) PvLEA-22 accurately reproduced the conformational changes of the native LEA protein in response to added salt. We conclude that both PvLEA-22 and the control peptide can sequester ions, although the efficiency may depend on the amino acid sequence of these peptides. Second, TMDSC measurements demonstrated that dried PvLEA-22 was in the glassy state at ambient temperatures, independent of the choice of the added salt. On the basis of these results, it can be inferred that the 11-mer motif units in G3LEA proteins are able to play two roles simultaneously: as an ion scavenger and as a biological glass-former.

## ASSOCIATED CONTENT

### Supporting Information

Functional properties of G3LEA proteins under high-salt conditions (Table S1); data for the water content dependence of the thermodynamic and secondary structural parameters for PvLEA-22 mixed with no salts, NaCl [0.5], and NaCl [1.0]

(Tables S2, S3, and S4); data for band positions and relative areas of the FT-IR spectra for PvLEA-22 (D) and the control peptide (D) dried with a selected salt (Table S5). This material is available free of charge via the Internet at <http://pubs.acs.org>.

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## ABBREVIATIONS

G3LEA, group 3 late embryogenesis abundant; FT-IR, Fourier transform infrared; TMDSC, temperature-modulated differential scanning calorimetry; PvLEA-22, 22-mer peptide constructed as the model peptide of the LEA protein originating from an insect, *Polypedium vanderplanki*;  $T_g$ , glass transition temperature.

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**Table S1.** Functional properties of G3LEA proteins under high-salt conditions.

Organism	Expressed G3LEA (Source)	Stress	Findings under the given stress <sup>1)</sup>	Ref.
<i>E. coli</i>	PM2 (soybean)	0.5 M NaCl or KCl (LB solid plate)	A	37
<i>E. coli</i>	PM2 (soybean)	0.12 M MgCl <sub>2</sub> or CaCl <sub>2</sub> (LB solid plate)	A	38
<i>E. coli</i>	PM30 (soybean)	0.8 M NaCl or 0.7 M KCl (LB liquid media)	B	39
<i>Saccharomyces cerevisiae</i>	HVA1 (barley)	1.2M NaCl or KCl (liquid media)	C	40
Rice	HVA1 (barley)	0.2 M NaCl (soil)	D	41
Rice	HVA1 (barley)	0.2 M NaCl (soil)	E	42
Radish	ME-LEA4 ( <i>B. napus</i> )	0.05 – 0.15 M NaCl (solid media)	F	43
Chinese cabbage	ME-LEA4 ( <i>B. napus</i> )	0.05 – 0.2 M NaCl (solid media)	G	44

1) The letter in the column corresponds to the following summaries of the experimental results, respectively:

- A. The recombinant strain had higher survival rates than the control strain.
- B. The recombinant strain showed improved growth in comparison with the control strain.
- C. The recombinant yeast exhibited better growth performance than the control strain.
- D. The transgenic rice plant grew faster than the non-transgenic plant.
- E. The transgenic rice seedlings showed enhanced growth relative to those of a control plant.
- F. The transgenic radish plant had a higher growth rate than the non-transgenic plant.
- G. The transgenic Chinese cabbage showed a higher seed germination rate than the non-transgenic plant.

**Table S2.** Water Content Dependence of Physicochemical Properties of Salt-free PvLEA-22.

Moisture condition (RH / %) <sup>1)</sup>	Water content		T <sub>g</sub> / °C	$\Delta H_{\text{endotherm}}$ <sup>2)</sup> / J g <sup>-1</sup>	$\alpha$ -helix / cm <sup>-1</sup>
	/ wt%	H <sub>2</sub> O moles / PvLEA-22 mole			
5 <sup>3)</sup>	2	3	108	N.D.	1653
38 (6 h) <sup>4)</sup>	7	9	-10	N.D.	1653
60 (4 h) <sup>4)</sup>	9	12	-11	N.D.	1653
75 (3 h) <sup>4)</sup>	12	17	-17	0.2 ( -4 to 6 °C) <sup>5)</sup>	1653
85 (1 h) <sup>4)</sup>	15	21	-19	0.3 ( -5 to 9 °C) <sup>5)</sup>	1652
93 (15 min) <sup>4)</sup>	20	30	-28, 5 <sup>6)</sup>	N.D.	1652
93 (30 min) <sup>4)</sup>	~30	52	7)	7)	N. D.

1) The numbers in parentheses is the time of moisture treatment.

2) Enthalpy observed for the endothermic peak around 0 °C.

3) This sample corresponds to sample A in Table 1.

- 4) Time required until the water content reaches a steady value under the given RH condition.
- 5) The temperature range of the observed endothermic peak.
- 6) Two glass transitions were observed, due to phase separation under cooling.
- 7) No TMDSC measurements.

**Table S3.** Water Content Dependence of Physicochemical Properties of PvLEA-22 mixed with NaCl [0.5].

Moisture condition (RH /%) <sup>1)</sup>	Water content		T <sub>g</sub> / °C	$\Delta H_{\text{endotherm}}$ <sup>3)</sup> / J g <sup>-1</sup>	$\alpha$ -helix / cm <sup>-1</sup>
	/ wt%	H <sub>2</sub> O moles / Solute <sup>2)</sup> mole			
5 <sup>4)</sup>	2	3	99	N.D.	1654
38 (8 h) <sup>5)</sup>	7	12	-10	N.D.	1654
60 (10 h) <sup>5)</sup>	9	16	-10	N.D.	1654
75 (30 min) <sup>5)</sup>	16	30	-23	N.D.	1653
75 (1.5 h) <sup>5)</sup>	23	47	-31, 3 <sup>6)</sup>	N.D.	1653
75 (20 h) <sup>5)</sup>	~40	105	7)	7)	N.D.

1) The numbers in parentheses is the time of moisture treatment.

2) Solute means the mixture of PvLEA-22 1mol and the added NaCl.

3) Enthalpy observed for the endothermic peak around 0 °C.

4) This sample corresponds to sample **B** in Table 1.



5) Time required until the water content reaches a steady value under the given RH condition.

6) Two glass transitions were observed, due to phase separation under cooling.

7) No TMDSC measurements.

**Table S4.** Water Content Dependence of Physicochemical Properties of PvLEA-22 mixed with NaCl [1.0].

Moisture condition (RH /%) <sup>1)</sup>	Water content		T <sub>g</sub> / °C	$\Delta H_{\text{endotherm}}$ <sup>3)</sup> / J g <sup>-1</sup>	$\alpha$ -helix / cm <sup>-1</sup>
	/ wt%	H <sub>2</sub> O moles / Solute <sup>2)</sup> mole			
5 <sup>4)</sup>	4	8	95	N.D.	1658
38 (6 h) <sup>5)</sup>	6	12	-9	N.D.	1658
60 (9 h) <sup>5)</sup>	9	19	-11	N.D.	1657
75 (1 h) <sup>5)</sup>	19	45	N.D.	N.D.	1657
75 (3 h) <sup>5)</sup>	31	87	N.D.	N.D.	1657
75 (6h) <sup>5)</sup>	40	129	6)	6)	N.D.
75 (20 h) <sup>5)</sup>	~48	178	6)	6)	N.D.

1) The numbers in parentheses is the time of moisture treatment.

2) Solute means the mixture of PvLEA-22 1mol and the added NaCl.

3) Enthalpy observed for the endothermic peak around 0 °C.

- 4) This sample corresponds to sample *C* in Table 1.
- 5) Time required until the water content reaches a steady value under the given RH condition.
- 6) No TMDSC measurements.

**Table S5.** Peak Positions (cm<sup>-1</sup>) of the Spectral Components of the Amide I Band and Their Relative Areas (%) <sup>1)</sup> in Deuterated Model Peptides Dried with or without a Selected Salt.

Chemical components <sup>2)</sup>	Spectral components													
	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%	cm <sup>-1</sup>	%
<b>PvLEA-22 (D)</b>														
No salts <sup>3)</sup>	1618	18	1630	23	1643	15	1659	20	1674	25				
NaCl [0.5]	1615	4	1628	11	1641	14	1654	23	1672	25	1689	24		
NaCl [1.0]			1627	8	1638	6	1651	30	1673	27	1681	12	1694	17
KCl [0.5]	1615	8	1628	10	1641	12	1655	18	1670	24	1688	28		
KCl [1.0]			1627	16	1639	13	1653	20	1668	15	1679	17	1693	19
MgCl <sub>2</sub> [0.5]			1633	47			1656	20	1673	12	1687	21		
MgCl <sub>2</sub> [1.0]			1635	51			1660	21	1674	7	1687	21		
CaCl <sub>2</sub> [0.5]			1628	39	1647	21			1668	23	1680	4	1695	15
CaCl <sub>2</sub> [1.0]			1626	35	1646	24			1665	16	1678	11	1697	14
<b>Control (D)</b>														
No salts					1639	48			1672	52				
NaCl [0.5]			1630	21	1639	26			1672	34	1678	7	1695	13
NaCl [1.0]			1628	24	1639	15	1651	12	1663	13	1676	16	1691	20
KCl [0.5]			1630	24	1641	25			1665	28	1680	7	1693	17
KCl [1.0]			1628	26	1640	13	1651	10	1663	16	1677	17	1692	18
MgCl <sub>2</sub> [0.5]			1627	28	1639	9	1646	15	1667	20	1678	13	1694	15
													(Continued)	



Table S2 (cont'd)														
MgCl <sub>2</sub> [1.0]	1619	20			1633	30			1661	21	1676	15	1694	15
CaCl <sub>2</sub> [0.5]	1609	7	1623	15	1635	16	1648	20	1664	16	1679	13	1697	13
CaCl <sub>2</sub> [1.0]	1610	3	1628	13	1639	39			1664	28	1676	5	1697	12

- 1) Numerical data of relative areas were rounded to one decimal place and thereby the sum of the relative areas over all amide I components is not necessarily 100 %.
- 2) 0.5 and 1.0 in the bracket indicate the molar ratios of the added salt per amino acid residue of the each peptide.
- 3) All numerical data were cited from our previous paper (Ref.29).